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Resumo

A doença de Alzheimer (AD) é a forma mais comum de demência, constituindo um problema de saúde pública grave, devido ao aumento da esperança média de vida da população mundial, bem como ao longo período de progressão desta doença incapacitante. A AD é caracterizada, em termos patológicos, por acumulação de péptido amilóide-β (Aβ), a nível extracelular e de tranças neurofibrilares intraneuronais, compostas por agregados de proteína tau hiperfosforilada, em regiões cerebrais específicas. A neuroinflamação crónica e a perda progressiva de neurónios, sinapses e matéria branca são características adicionais que também estão associadas à doença.

O ácido tauroursodesoxicólico (TUDCA) é um ácido biliar endógeno, anti-apoptótico, capaz de atravessar a barreira hematoencefálica e que apresenta fortes propriedades neuroprotectoras, em diversos modelos experimentais de AD. Resultados obtidos anteriormente pelo nosso grupo demonstraram a eficácia terapêutica do TUDCA em murganhos duplamente transgénicos APP/PS1, antes de estes apresentarem deposição de placas amilóides. Neste estudo, o tratamento preventivo com TUDCA resultou, não só na diminuição da produção e acumulação de Aβ, como também na redução da activação da resposta glial face à acumulação de agregados amilóides. É de salientar que o tratamento com TUDCA conduziu a melhorias na memória dos animais, o que poderá estar relacionado com a diminuição da perda de sinapses, também observada.

No estudo agora apresentado, pretendeu-se avaliar os efeitos protetores do TUDCA, quando administrado após o desenvolvimento da patologia amilóide no mesmo modelo transgénico de AD. Os murganhos APP/PS1 com 7 meses de idade foram injetados intraperitonealmente com TUDCA (500 mg/kg), a cada 3 dias, durante 3 meses. Após a realização do teste aquático de Morris, destinado à avaliação da memória espacial dos murganhos, os animais foram sacrificados e um dos hemisférios cerebrais foi processado para análise imunohistoquímica (IHC). O outro hemisfério foi preservado para isolamento do hipocampo e córtex frontal e posterior extração de RNA e proteínas.

O tratamento com TUDCA levou a uma diminuição significativa da deposição de Aβ em ambas as regiões cerebrais analisadas, com uma maior redução detetada no hipocampo. Esta observação explica-se, provavelmente, pelo facto de neste modelo transgénico, o hipocampo apresentar placas amilóides apenas a partir dos 2-3 meses, enquanto que o neocórtex apresenta depósitos de Aβ logo a partir das 6 semanas. Contudo, ensaios de ELISA confirmaram que os níveis de $A\beta_{40}$ e $A\beta_{42}$ estavam significativamente diminuídos em ambas as regiões, após o tratamento com TUDCA. Estes resultados estão de acordo com a diminuição do processamento amiloidogénico da proteína precursora do Aβ (APP) observada no córtex e hipocampo, e sugerem que o tratamento com TUDCA interfere na produção de Aβ.

Avaliaram-se também os níveis de fosforilação de resíduos específicos da proteína cinase B, ou Akt, e da cinase da glicogénio sintase 3β (GSK3β), que se relacionam indiretamente com os seus níveis de atividade. A enzima Akt, quando ativa, encontra-se fosforilada no resíduo de serina 473, podendo então fosforilar a GSK3β na serina 9, o que inibe a atividade desta. É de salientar que o eixo Akt/GSK3β se encontra desregulado na AD. A enzima Akt é alvo da via controlada pela fosfatidilinositol-3-cinase (PI3K), estando ambas envolvidas na ativação de mecanismos de sobrevivência neuronal e de potenciação sinática. A GSK3β encontra-se amplamente descrita como estando hiperativada na AD, ,parecendo envolvida em inúmeros processos associados à progressão da doença. Por exemplo, a GSK3β é uma das principais enzimas responsáveis pela fosforilação de vários resíduos da proteína tau, pelo que a sua desregulação se encontra associada à hiperfosforilação da tau. Mais ainda, a GSK3β tem a capacidade de fosforilar a APP num resíduo citoplasmático, o que pode contribuir para a produção de Aβ. Curiosamente, a inibição da atividade desta cinase parece diminuir a expressão da β-secretase (BACE1), reduzindo o processamento amiloidogénico da APP e, consequente, a produção de Aβ. A GSK3β surgiu recentemente como um mediador-chave da ativação microglial e astroglial em condições inflamatórias, incluindo na AD. Neste sentido, a inibição desta cinase *in vivo* tem o potencial de melhorar significativamente as patologias associadas à AD.

Como seria de esperar, a análise por Western blot (WB) revelou uma diminuição dos níveis de fosforilação da Akt e da GSK3β no córtex, bem como uma

diminuição nos níveis de fosforilação da GSK3β no hipocampo de murganhos APP/PS1. Em contrapartida, os animais transgénicos tratados com TUDCA apresentaram níveis de fosforilação destas enzimas semelhantes aos detetados nos murganhos controlo, o que sugere que o TUDCA normaliza os níveis de atividade destas enzimas, no contexto da AD. Os níveis de fosforilação do resíduo de serina 396 da proteína tau, que se encontram aumentados nos murganhos APP/PS1, foram igualmente diminuídos pelo tratamento com TUDCA. Como este resíduo é um alvo importante da GSK3β, a diminuição dos seus níveis de fosforilação poderão indicar a reposição da atividade da GSK3β pelo TUDCA. Verificou-se ainda que, ao contrário do esperado, o tratamento com TUDCA não afetou o nível de fosforilação da APP e a quantidade de BACE1 presente nos cérebros dos murganhos transgénicos tratados, face ao observado nos murganhos transgénicos não tratados, indicando que a atividade desregulada da GSK3β não modula estas vias neste modelo animal, ou que outros mecanismos moleculares poderão estar envolvidos.

Neste estudo, e à semelhança de outros anteriores, o tratamento com TUDCA atenuou significativamente a ativação pro-inflamatória dos astrócitos e da microglia em redor das placas amilóides. De igual modo, o tratamento com TUDCA conduziu a uma diminuição significativa dos níveis de mRNA do factor de necrose tumoral α (TNFα), uma das citocinas pro-inflamatórias aumentadas na AD e envolvidas na progressão da doença.

Relativamente ao conteúdo sinático, detetou-se uma diminuição nos níveis da proteína sinaptofisina no giro denteado do hipocampo de murganhos APP/PS1, que se correlaciona com a disfunção destas estruturas e a desregulação neurológica generalizada. Esta redução foi parcialmente revertida nos animais tratados com TUDCA, podendo dever-se ao aumento de atividade da cinase Akt que se encontra envolvida na potenciação da resposta sinática ou, simplesmente, à diminuição dos níveis de Aβ. Contudo, os estudos comportamentais permitiram concluir que o tratamento tardio com TUDCA, apesar de melhorar alguns aspetos da patologia, ao nível molecular, não melhora significativamente a perda de memória espacial dos murganhos APP/PS1, tendo-se observado apenas uma tendência para tal. Esta observação poderá advir do facto destes animais já manifestarem défices de memória desde os 7 meses de idade, altura em que se iniciou o tratamento com TUDCA.

Em conclusão, os resultados obtidos neste trabalho demonstraram a eficácia terapêutica do TUDCA quando administrado a murganhos APP/PS1 já com características patológicas associadas à AD. O TUDCA atenua a produção e deposição de Aβ, a hiperfosforilação da proteína tau, a activação glial e a perda de integridade sináptica, sendo que vários destes efeitos poderão dever-se, especificamente, à modulação da via Akt/GSK3β. Estes dados sugerem que a utilização do TUDCA não reverte as condições patológicas associadas à perda de memória emergentes ou já existentes na AD sendo, no entanto, uma estratégia terapêutica promissora para atenuar ou retardar a progressão da doença.

Palavras-chave: Amilóide-β; Cinase da glicogénio sintase 3β; Murganho APP/PS1; Neuroinflamação; Proteína precursora do amilóide- β; Tau; TUDCA

Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder hallmarked by the accumulation of extracellular amyloid-β (Aβ) peptide and intraneuronal neurofibrillary tangles composed of hyperphosphorylated tau, as well as chronic neuroinflammation and loss of neurons, synapses and white matter. Tauroursodeoxycholic acid (TUDCA) is an endogenous anti-apoptotic bile acid with potent neuroprotective properties in several experimental models of AD. We have previously reported the therapeutic efficacy of TUDCA treatment before amyloid plaque deposition in APP/PS1 double-transgenic mice. Importantly, preventive treatment with TUDCA not only reduced Aβ generation and accumulation, while reducing gliosis, but also ameliorated memory deficits and protected against synaptic loss.

In the present study, we evaluated the protective effects of TUDCA when administrated after the onset of amyloid pathology. APP/PS1 transgenic mice with 7 months of age were injected intraperitoneally with TUDCA (500 mg/kg) every 3 days, for 3 months. TUDCA treatment significantly attenuated Aβ deposition in the brain, with a concomitant decrease in $A\beta_{40}$ and $A\beta_{42}$ levels. The amyloidogenic processing of the amyloid precursor protein was also reduced, indicating that TUDCA interferes with Aβ production. In addition, TUDCA abrogated GSK3β hyperactivity, which is highly implicated in tau hyperphosphorylation and glial activation. This effect was likely dependent on the specific activation of the upstream kinase, Akt. TUDCA treatment also reduced microglial and astroglial activation, and tumor necrosis factor α (TNFα) expression levels. Finally, TUDCA treatment partially rescued synaptic loss in the dentate gyrus of the hippocampus. Overall, our results suggest that TUDCA is a promising therapeutic strategy not only for the prevention but also for the treatment of AD after disease onset.

Keywords: Amyloid-β; Amyloid precursor protein; APP/PS1 mice; Glycogen synthase kinase β; Neuroinflammation; Tau; TUDCA

Abbreviations

Aβ	Amyloid-β
Aβo	Aβ oligomers
AD	Alzheimer's disease
ADAM	A disintegrin and metalloproteinase
ANOVA	Analysis of variance
AP-1	Activator protein 1
APOE	Apolipoprotein E
APP	Amyloid precursor protein
BACE1	Beta-site APP cleaving enzyme 1
CD	Cluster of differentiation
Cdk 5	Cyclin-dependent kinase 5
CEBPD	CCAAT/enhancer-binding protein delta
CNS	Central nervous system
CREB	cAMP response element binding protein
CSF	Cerebrospinal fluid
CT	Computed tomography
CTF	C-terminal fragment
Cyt *c*	Cytochrome *c*
DAMPs	Damage-associated molecular patterns
ELISA	Enzyme-linked immunosorbent assay
EOAD	Early onset Alzheimer's disease
ER	Endoplasmic reticulum
fAβ	Fibrillar Aβ
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GSK3	Glycogen synthase kinase 3
GUDCA	Glycoursodeoxycholic acid
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IGF	Insulin like growth factor 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IR	Insulin receptor
IRS	Insulin receptor substrate

JNK	c-Jun N-terminal kinase
LOAD	Late onset Alzheimer's Disease
LTD	Long Term depression
LTP	Long term potentiation
MAP	Microtubule associated proteins
MAPK	p38 Mitogen-activated protein kinase
MCP1	Monocyte chemoattractant protein 1
MCI	Mild cognitive impairment
MGV	Mean gray values
MPT	Mitochondrial permeability transition
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin kinase
mTORC2	Mammalian target of rapamycin-2
NF-κB	Nuclear factor κB
NFT	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
NSAID	Nonsteroidal anti-inflammatory drug
NSR	Nuclear steroid receptors
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
p-APP	Phosphorylated APP at Thr668
PDK1	Phosphoinositide-dependent protein kinase-1
PET	Positron emission tomography
PI3K	Phosphatidylinositide 3-'OH kinase
PIP3	Phosphatidylinositol-3,4,5-Trisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PP	Protein phosphatase
PS1	Presenilin 1
PS1	Presenilin 2
PSD-95	Postsynaptic density-95
qRT-PCR	Quantitative real-time PCR
RAGE	Receptor for advanced glycation end-products
RM	Repeated measures
ROS	Reactive oxygen species

sAPP	soluble APP
SEM	Standard error of the mean
SP	Senile plaque
STAT3	Signal transducer and activator of transcription 3
SRA	Scavenger receptor A-1
SYN	Synaptophysin
TGFβ	Transforming growth factor β
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
TNFR	TNF-α receptor
TUDCA	Tauroursodeoxycholic acid
UDCA	Ursodeoxycholic acid
Wt	Wild type

I. INTRODUCTION AND OBJECTIVES

Dementia is nowadays a major public health problem, since overall life expectancy has greatly increased. Worldwide, dementia was estimated to affect more than 35.6 million individuals in 2010, and is expected to double in the next 20 years (Prince et al., 2013). Alzheimer's disease (AD) accounts for approximately 70% of the cases associated with dementia, thus being the most common manifestation of this type of pathology (Reitz and Mayeux, 2014).

1. Alzheimer's Disease

Symptomatically, AD is initially manifested as a progressive loss of episodic memory – termed mild cognitive impairment (MCI) – that slowly progresses toward full dementia, characterized by extensive memory deterioration, cognitive impairment and abnormal behavior (Selkoe, 2001; Reitz and Mayeux, 2014). AD is a disabling, slow progressing disease, usually developing over the course of decades. Consequently, AD patients become highly demanding cases on healthcare and social services, with a huge impact in the countries' economies (Reitz and Mayeux, 2014).

At early stages of AD, the main affected brain areas are the cholinergic basal forebrain and medial temporal lobe structures, including the hippocampus and entorhinal cortex. With disease progression, several cortical regions become also affected (Braak and Braak, 1991).

Pathologically, AD is hallmarked by the presence of extracellular senile plaques (SPs) composed of amyloid-β (Aβ) peptide aggregates, intraneuronal neurofibrillary tangles (NFT) composed of hyperphosphorylated tau protein, chronic neuroinflammation and loss of neurons, synapses and white matter (Fig. 1) (Selkoe, 2001). Since AD is the most common form of dementia, the etiology and pathology mechanisms of this devastating disease have been intensively investigated after its discovery, in 1906 (Selkoe, 2001). However, the triggering mechanisms of AD are far from fully deciphered, and effective diagnosis and treatment are still highly demanded.

1.1. Epidemiology and risk factors

Advanced age is the most pronounced risk factor for AD, with the prevalence of the disease increasing substantially between the 65 and 85 years of age, so that AD affects 30–50% of all people with 85 years (Isik, 2010; Reitz and Mayeux, 2014).

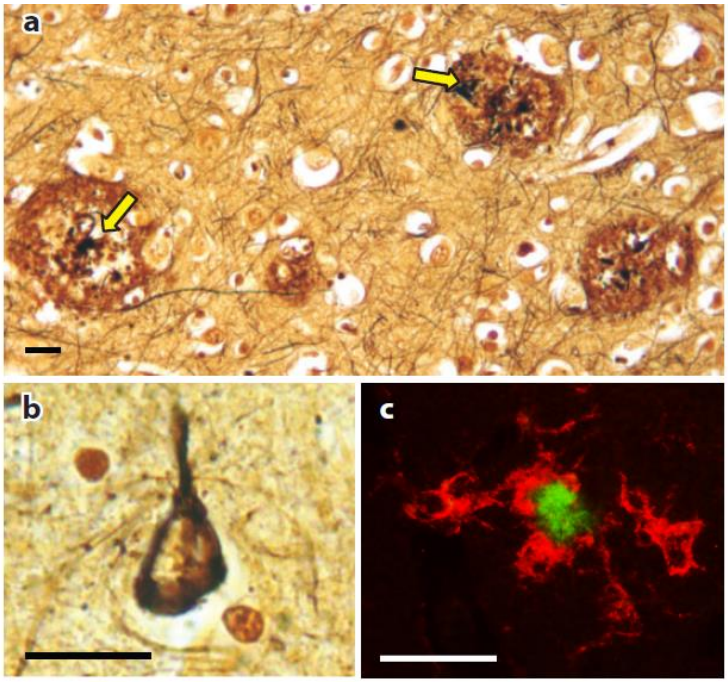


Fig. 1. Neuropathological hallmarks of Alzheimer's disease. (A, B) Silver staining for extracellular amyloid plaques (*yellow arrows*) (A) and intraneuronal neurofibrillary tangles (B) in brain sections from an AD patient. (C) Immunohistochemical staining of a plaque composed of Aβ (*green*), presenting infiltrating filaments from microglia, here stained with an anti-Iba-I antibody (*red*). *Scale bar*, 40 μm. Adapted from O'Brien and Wong, 2011.

Based on the age of onset, the disease can be classified as early onset AD (EOAD), if it occurs before 65 years of age, and late onset AD (LOAD), after 65 years (Isik, 2010).

EOAD, which is clinically indistinguishable from LOAD, accounts for 1-5% of all cases and is usually inherited in an autosomal dominant manner, being therefore termed familial AD. EOAD results from mutations in three different genes, the *amyloid precursor protein* (*APP*) gene and the *presenilin 1* (*PS1*) and *presenilin 2* (*PS2*) genes, with mutations in the *PS1* gene accounting for most of the cases (Selkoe, 2001; Reitz and Mayeux, 2014).

LOAD typically correlates with sporadic forms of AD, and its causative factors are still unclear. Like most chronic diseases, AD appears to be a multifactorial disease. Epidemiological studies have reported several possible risk factors that can be associated with LOAD etiology. These include limited intellectual and cognitively stimulating activities at both young and old ages, low levels of education and occupation, lack of physical exercise during late life, cerebrovascular diseases,

traumatic brain injuries, hypo- and hypertension, diabetes and obesity (Reitz and Mayeux, 2014).

Although environmental factors may alter the susceptibility to LOAD, several genome-wide association studies (GWAS) have implicated putative genetic factors that contribute to the onset of LOAD (Reitz and Mayeux, 2014), including the presence of the ε4 allele of *apolipoprotein E* (*APOE*) gene. The protein encoded by this allele appears to be structurally altered, which results in deregulated brain lipid metabolism and abnormal interaction of ApoE with Aβ peptides, leading to reduced Aβ clearance and enhanced Aβ aggregation (Bu, 2009). Several other *loci* have been proposed to influence the susceptibility to AD based on GWAS studies. Curiously, the vast majority are clustered in four main pathways, namely immune response, APP processing, lipid metabolism, and endocytosis (Reitz and Mayeux, 2014).

1.2. Pathogenesis

The long pre-clinical phase of AD encompasses several pathologic mechanisms in specific brain regions that primarily lead to synaptic loss, the first culprit involved in memory and cognitive deficits in AD patients, followed by neurodegeneration and development of SPs and NFTs, the histopathological features of AD (Selkoe, 2001; Walsh and Selkoe, 2007; Perrin et al., 2009). These pathological mechanisms involve ROS production, mitochondrial failure, deregulation of Ca^{2+} metabolism, inflammation and neuronal dysfunction (Cavallucci et al., 2012).

1.2.1. Amyloid cascade hypothesis

Aβ peptides are the principal constituents of SPs found in AD brains (Selkoe, 2001). Aβ derives from the sequential proteolysis of APP, a single-pass transmembrane glycoprotein with a large extracellular domain (O'Brien and Wong, 2011; Haass et al., 2012). Depending on its subcellular location, APP can be preferably cleaved through the amyloidogenic or anti-amyloidogenic pathways (Fig. 2) (O'Brien and Wong, 2011; Haass et al., 2012).

In the amyloidogenic pathway, APP processing is mainly catalyzed by the canonical β-secretase beta-site APP cleaving enzyme 1 (BACE1), a transmembrane aspartic protease that presents optimal activity in slightly acidic compartments, such as

endocytic vesicles and endosomes. Cleavage of APP by β-secretase leads to the shedding of its large ectodomain (termed soluble APP-β fragment, or sAPP-β) and the production of a small membrane-spanning C-terminal fragment β (CTF-β). CTF-β is further cleaved by γ-secretase in the transmembrane domain, generating Aβ and releasing the C-terminal fragment γ (CTF-γ) into the cytoplasm (O'Brien and Wong, 2011; Haass et al., 2012).

Alternatively, APP can be processed by α-secretase activity, catalyzed by the ADAM (a disintegrin and metalloproteinase) family of proteases that occurs mainly on the cellular membrane and generates soluble sAPPα and APP C-terminal fragment α (CTF-α). The α-secretase cleavage site is located within the Aβ region, thus precluding BACE1 cleavage and Aβ production. Finally, cleavage by γ-secretase releases CTF-γ and a truncated Aβ peptide termed p3, which apparently does not have any biological relevance. Importantly, γ-secretase can cleave CTFs by performing sequential series of intramembranous cuts, thus yielding Aβ peptides with approximately 4 kDa and lengths that range from 38 to 43 amino acids. The most common form of Aβ contains 40 amino acids ($A\beta_{40}$), while the most toxic contains 42 amino acids in length ($A\beta_{42}$) (Selkoe, 2001; O'Brien and Wong, 2011; Haass et al., 2012).

Curiously, Aβ production occurs constitutively in the brain throughout life, and the peptide is suggested to have physiological roles at very low concentrations. In fact, Aβ potentiates hippocampal long-term potentiation (LTP) at picomolar concentrations (Puzzo et al., 2008). Moreover, Aβ is also suggested to possess limited antioxidant activities due to its ability to chelate redox metal ions (Atwood et al., 2003). Finally, Aβ appears to present neurotrophic properties under deprived conditions (Fonseca et al., 2013).

Conversely, at supraphysiological concentrations, Aβ self-aggregates into higher order structures. Initially, soluble Aβ acquires higher β-sheet content, leading to self-dimerization/trimerization, which is followed by oligomerization into soluble high molecular aggregates forming spherical oligomers, protofibril formation and finally aggregation into insoluble fibrillar structures characteristic of SPs (Serpell, 2000; Kayed and Lasagna-Reeves, 2013). Importantly, mounting evidence indicates that increases in the ratio $A\beta_{42}/A\beta_{40}$ within the brain is predictive of AD progression, rather than the total levels of Aβ species (Jarrett et al., 1993). This reflects the higher hydrophobic

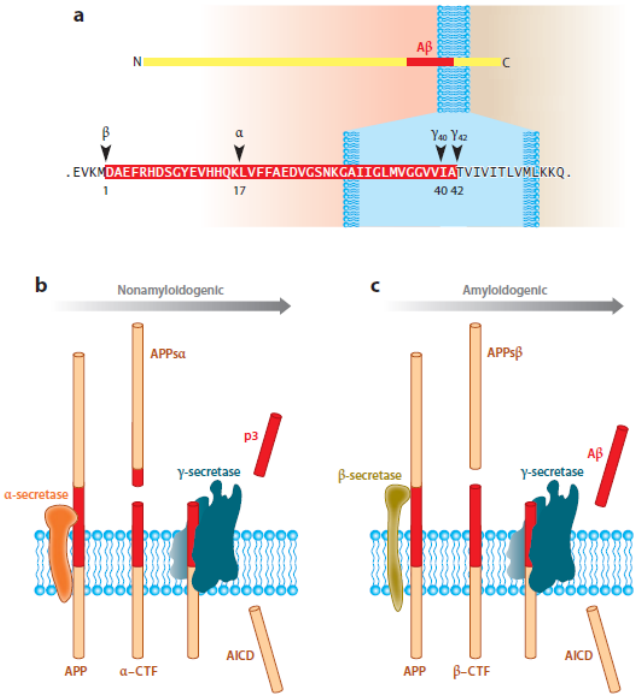


Fig. 2. APP processing pathways. (A) APP presents a large N-terminal ectodomain, which contains a portion of Aβ (*red*), while the remaining portion of the peptide is contained within the transmembrane region. The cleavage sites for α- and β-secretases, as well as the the two most common cleavage sites for γ-secretase, are shown in the peptide sequence. (B) Non-amyloidogenic processing of APP by α- and γ-secretases, resulting in the production of sAPP α, CTF-α and ultimately CTF- γ and p3. (C) Amyloidogenic processing of APP by β- and γ-secretases, which generates sAPP-β and a membrane tethered CTF-β that is further cleaved by γ-secretase, yielding Aβ and CTF- γ. Adapted from O'Brien and Wong, 2011.

nature of $A\beta_{42}$, more prone to aggregation. In fact, $A\beta_{42}$ is the main Aβ species detected at the core of amyloid plaques, and potentially the first form to aggregate, then triggering the misfolding of other Aβ species (Iwatsubo et al., 1994; Selkoe, 2001).

The prominent role of Aβ in AD pathological events culminates in the construction of the amyloid cascade hypothesis. This hypothesis advocates that augmented Aβ generation and/or impaired Aβ clearance contributes to increased amounts of Aβ in the brain parenchyma, which then aggregate into higher assembly states that constitute the main pathological agent involved in AD progression, ultimately leading to neurodegeneration and dementia (Selkoe, 2001). The amyloid cascade hypothesis is strongly supported by the finding of familial AD-related mutations in the genes coding for APP and for the catalytic subunits of γ-secretase, PS1 and PS2,

which are respectively associated with increased amyloidogenic processing of APP and preferential production of longer Aβ species with higher amyloidogenic propensity, such as $A\beta_{42}$ (Selkoe, 2001; Reitz and Mayeux, 2014).

In sporadic forms of AD, increasing evidence implicates impaired Aβ clearance as the initial event leading to Aβ accumulation. For example, ApoE ε4 isoform is associated with defective Aβ clearance (Bu, 2009). Furthermore, the activities of major Aβ-degrading enzymes, including neprilysin and insulin-degrading enzyme, have also been reported to be diminished with age and in AD brains (Wang et al., 2006).

Since Aβ plaques are a major hallmark of AD, focus was initially given to the toxic properties of fibrillar Aβ (fAβ), the main constituent of SPs. Curiously, despite some early studies reporting neurotoxic effects following fAβ exposure (Lorenzo and Yankner, 1994; Geula et al., 1998), it is now established that the presence of amyloid plaques do not directly correlate with cognitive decline in AD patients, while healthy aged individuals sometimes present high plaque number with no AD symptomology (Naslund et al., 2000; Cavallucci et al., 2012). However, amyloid plaques can be the cause of indirect toxicity, since the presence of SPs is a key event in glial activation that drives neuroinflammation, other typical feature involved in AD progression (Meraz-Rios et al., 2013).

These results propelled further investigation, which ultimately revealed that higher levels of soluble Aβ oligomers (Aβo) show a robust correlation with extensive synaptic loss, as well as with cognitive deficits (Walsh and Selkoe, 2007; Cavallucci et al., 2012). Further studies have identified several structurally diverse Aβo, usually formed from 9 to 50 molecules of the peptide that induce toxicity and neuronal dysfunction when administrated to neuronal cultures and mouse brains (Cavallucci et al., 2012; Kayed and Lasagna-Reeves, 2013).

Regarding direct synaptic loss, growing evidence suggests that soluble forms of Aβo might physically interact with postsynaptic receptors, such as the N-methyl-D-aspartate (NMDA) receptor, which limits Ca^{2+} influx through these receptors and triggers signalling cascades usually activated in long-term depression (LTD), a form of synaptic plasticity that reduces synaptic surface area and transmission (Shankar et al., 2007; Knobloch and Mansuy, 2008; Selkoe, 2008). Different species of extracellular Aβo have also been reported to bind to other cell surface receptors, such as the nerve

growth factor and insulin receptors, which can possibly induce aberrant cellular responses and ultimately neurotoxicity (Yaar et al., 1997; Zhao et al., 2008; Kayed and Lasagna-Reeves, 2013).

In addition to extracellular Aβ, there is evidence that Aβ in different assembly states can accumulate in several subcellular compartments, which further amplifies the plethora of pathological mechanisms attributed to Aβ (Kayed and Lasagna-Reeves, 2013). Multiple studies indicate that Aβo interact with biological membranes and induce their permeabilization and/or formation of ionic pores, which is connected to leakage of lysosomal content into the cytosol and disruption of Ca^{2+} homeostasis (Yang et al., 1998; Demuro et al., 2005; Kayed and Lasagna-Reeves, 2013). Aβ also induces endoplasmic reticulum (ER) stress, which further disrupts Ca^{2+} metabolism (Costa et al., 2010). Altered levels of intracellular Ca^{2+} then contribute to mitochondrial dysfunction, reactive oxygen species (ROS) production, deregulation of signalling pathways and synaptic dysfunction, ultimately leading to neurodegeneration (Yu et al., 2009).

Taking into account the central function of mitochondria in the cell, there is no doubt that mitochondrial dysfunction, abnormal mitochondrial dynamics and degradation by mitophagy occurring during aging play a key role in AD progression (Kayed and Lasagna-Reeves, 2013). Importantly, Aβ has been demonstrated to accumulate in brain mitochondria of both AD patients and mouse models, being detected before extensive amyloid deposition (Caspersen et al., 2005; Manczak et al., 2006; Dragicevic et al., 2010). Mitochondrial Aβ accumulation induced diminished enzymatic activity of respiratory chain complexes (III and IV) and subsequent decline in ATP production, while enhancing ROS production (Caspersen et al., 2005). Moreover, since synaptic function has a high energy demand, mitochondrial failure may have a major role in synaptic failure in AD. In fact, impaired synaptic plasticity in dentate granule cells is the earliest synaptic deficit in an AD mouse model, appearing to derive from mitochondrial dysfunction involving impaired Ca^{2+} uptake and increased ROS generation (Lee et al., 2012).

Finally, Aβ can impair glutamate reuptake by glial cells, which leads to excitotoxic mechanisms that injure nearby neurons and increase ROS production (Esposito et al., 2013). As previously indicated, ROS production is a common feature of

several deregulated pathways involved in AD, while levels of reactive nitrogen species (RNS) derived from reactive glial cells, are also elevated (Meraz-Rios et al., 2013). Mounting evidence demonstrates extensive oxidative damage, including lipid peroxidation and protein and nucleic acid oxidation, in vulnerable brain regions of AD patients (Bonda et al., 2010). Importantly, oxidative stress not only contributes to neuronal injury, as it may also potentiate Aβ production and aggregation. In this regard, increased ROS levels leads to increased translation of *BACE1* mRNA in the brains of AD patients and mouse models, thus enhancing the amyloidogenic processing of APP (Mouton-Liger et al., 2012). The development of such self-propelling cycles among Aβ-mediated aberrant mechanisms further hinders the isolation of the main pathological events involved in the etiology of AD.

Altogether, the aforementioned Aβ-driven pathologic mechanisms contribute to deregulation of numerous signalling pathways in the brain, thus unbalancing activities of kinases and phosphatases. The deregulation of multiple signalling pathways then culminates in neuronal dysfunction and, ultimately, in neuronal cell death (Fig. 3).

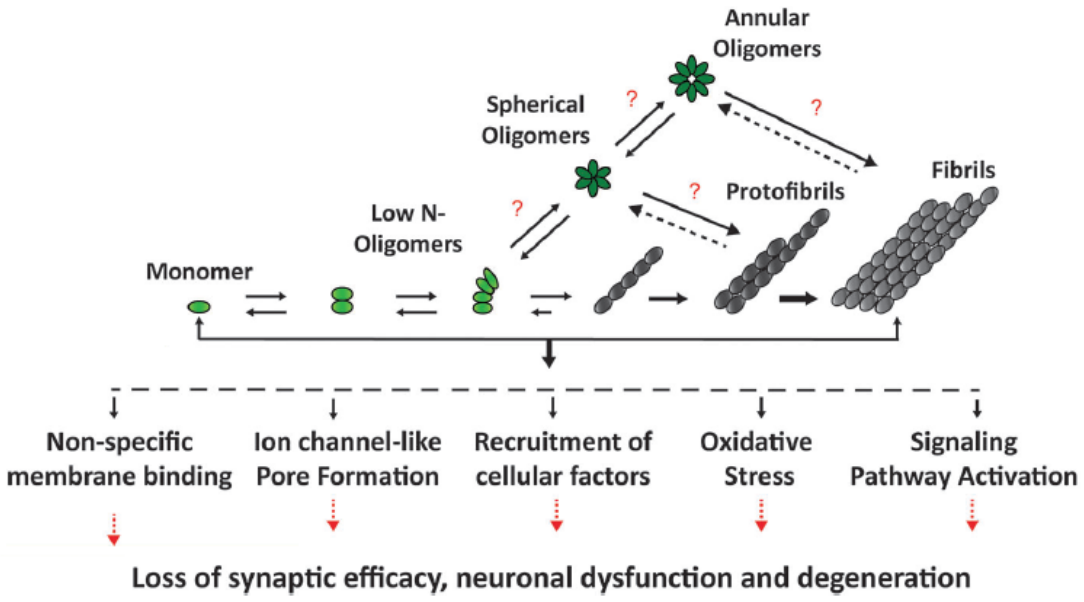


Fig. 3. Proposed neurotoxic mechanisms for aggregated Aβ species. At supraphysiological concentrations, Aβ can self-aggregate into higher order structures, ranging from low-n oligomers to insoluble fibrillar structures. Mounting evidence suggest that soluble oligomeric species of Aβ are the main toxic forms of the peptide, appearing to be involved in numerous neurotoxic mechanisms that culminate in neuronal dysfunction and death. Amyloid plaques, although appearing to lack direct neurotoxic effects beyond physically obstructing

neurites, are still important contributors to AD by eliciting a strong glial response leading to chronic neuroinflammation. Adapted from Kotler et al., 2014.

Regarding neurodegeneration, studies in the brain of AD patients showed a strong upregulation of apoptosis when compared to healthy age-matched controls, with activation of caspase-3 being detected in more than 50% of hippocampal neurons showing degenerated processes and co-localization with NFTs (Su et al., 2001; Colurso et al., 2003). Further, plentiful *in vitro* studies have demonstrated the apoptotic properties of Aβ species (Sola et al., 2003; Shi et al., 2010; Kong et al., 2013). These observations implicate apoptosis as a major form of Aβ-induced neuronal death.

1.2.2. Neurofibrillary tangles

In addition to Aβ plaques, neurofibrillary tangles are other typical histopathological feature of AD majorly constituted by hyperphosphorylated tau protein. Tau belongs to the family of microtubule-associated proteins (MAP), which are highly expressed in the CNS (Duan et al., 2012). Tau is mainly allocated to axons, where it binds to the microtubules to promote their assembly and stability. Further, binding of tau to the microtubules also regulates vesicular transport and axonal polarity. In the adult brain, tau is expressed as six different isoforms, ranging between 352 and 441 amino acid residues that derive from alternative splicing (Ballatore et al., 2007; Duan et al., 2012). When unphosphorylated, tau is very hydrophilic, presenting a natively unfolded structure in solution. The regulation of tau thus depends on conformational changes induced by residue-specific phosphorylations, with phosphorylation of more than 30 serine/threonine residues already described as modulating tau function (Stoothoff and Johnson, 2005). Importantly, tau promotes microtubule assembly when presenting a reduced phosphorylation state. The phosphorylation of tau in multiple residues has been attributed to several kinases, including glycogen synthase kinase 3β (GSK3β) and cyclin-dependent kinase 5 (CDK5). Conversely, to counterbalance kinase activity, numerous phosphatases can dephosphorylate tau residues, including protein phosphatase 1 (PP1), 2A (PP2A), 2B (PP2B) and 2C (PP2C) (Duan et al., 2012). Importantly, several tau-modifying kinases, such as GSK3β and CDK5, and phosphatases, such as PP2A, are consistently described as being deregulated in AD, thus leading to abnormal tau phosphorylation levels and subsequent tau malfunction

(Ballatore et al., 2007; Duan et al., 2012). In particular, phosphorylation of Ser205, Ser396, Ser404, Thr205 and Thr212 enhance tau polymerization into filaments and NFTs (Necula and Kuret, 2005; Li and Paudel, 2006; Duan et al., 2012). The direct role of tau aggregation in neurodegenerative processes are still not entirely understood, but it is known that tau dissociation destabilizes the axonal microtubule network, while toxic gain-of-function further drives tau aggregation. These processes ultimately result in impaired axonal transport and lead to synaptic failure and neurodegeneration (Fig. 4) (Ballatore et al., 2007).

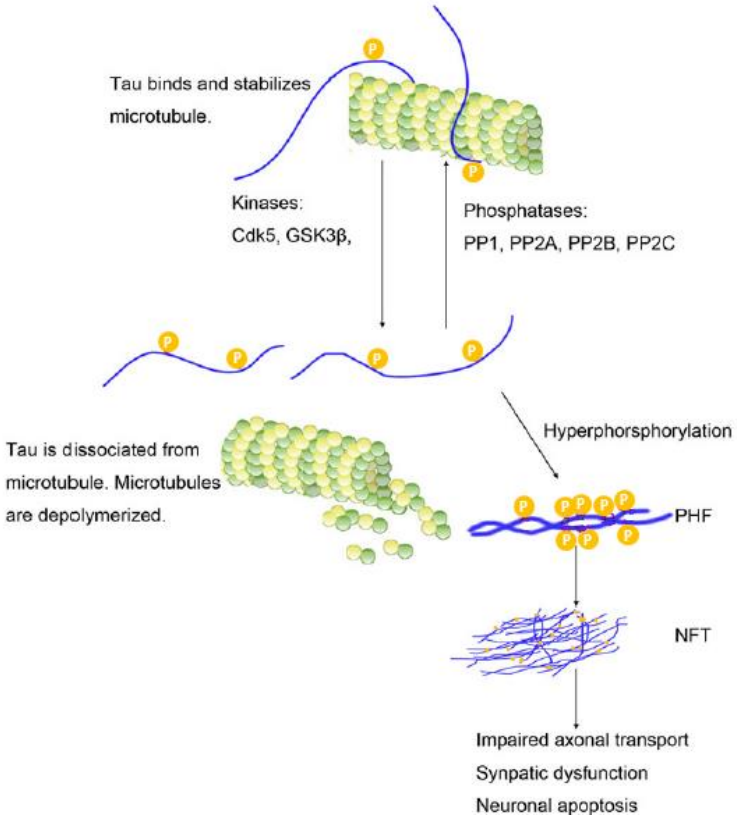


Fig. 4. Neurotoxicity mediated by tau dysfunction. During normal conditions, tau stabilizes microtubules through direct binding. This process is regulated by different tau phosphorylation patterns, which is tightly regulated by a large number of kinases and phosphatases. However, during AD, the activities of these enzymes are severely unbalanced, leading to tau hyperphosphorylation and dissociation from the microtubules. Hyperphosphorylated tau then aggregates into more organized forms until the formation of neurofibrillary tangles. Reduced functional tau function and physical obstruction by insoluble NFTs then lead to impaired axonal transport and subsequently to neuronal dysfunction and death. Adapted from Duan et al., 2012.

Supporting a prominent role for tau malfunction in dementia, mutations in the tau (*MAPT*) gene lead to the development of neurodegenerative disorders, termed tauopathies, such as the frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Ballatore et al., 2007). Moreover, unlike the distribution of amyloid plaques, the presence of tau pathology in AD is significantly correlated with neurodegeneration and the onset of clinical symptoms (Braak and Braak, 1991).

Amyloid plaques and NFTs are predominant pathologic features of AD that develop in the same brain regions, but the interacting mechanisms between these players are still unclear. Multiple lines of evidence suggest that Aβ acts upstream of tau pathology, since AD patients and mouse models develop deregulated activities of kinases and phosphatases involved in regulation of tau function before the appearance of tau pathology (Duan et al., 2012; Durairajan et al., 2012). Curiously, decreased endogenous tau levels prevent cognitive deficits in APP overexpressing mice, without altering amyloid pathology. In addition, APP/tau double transgenic mice present exacerbated tau pathology in areas with high levels of amyloid deposition when compared to single mutant tau transgenic mice, while amyloid deposition is not aggravated in the APP/tau mice as compared with single APP transgenic mice (Roberson et al., 2007; Duan et al., 2012). In vitro studies have also detected tau hyperphosphorylation in mature hippocampal neurons treated with Aβ, further supporting the view that NFT pathology is a downstream effect of Aβ accumulation (Ferreira et al., 1997; Duan et al., 2012).

2. Neuroinflammation in AD

Chronic neuroinflammation is also a characteristic feature of AD (Selkoe, 2001; Hickman et al., 2008; Lyman et al., 2013). It is well established that Aβ aggregates can elicit the development of inflammatory processes and increased gliosis, culminating in the abundant presence of activated microglia and reactive astrocytes in the close vicinity of amyloid plaques (Perlmutter et al., 1990; Hickman et al., 2008). During AD progression, Aβ continues to accumulate, leading to prolonged pro-inflammatory signalling to glial cells. Upon activation, glial cells secrete pro-inflammatory molecules, including cytokines, chemokines, free radicals, prostaglandins and complement factors, which in turn induce their own expression, in a positive feedback loop (Meraz-Rios et

al., 2013). This mechanism further exacerbates inflammation, which favors the amyloidogenic processing of APP, accumulation of Aβ and increased tau hyperphosphorylation, ultimately contributing to neurodegeneration. Increased Aβ levels potentiate the inflammatory response as well, leading to the generation of self-propelling cycles (Mandrekar-Colucci and Landreth, 2010; Meraz-Rios et al., 2013).

2.1. Microglia

Microglia are the resident macrophages of the brain and spinal cord, constituting the main line of the innate immune defense in the central nervous system (CNS). In humans, microglia comprise up to 16% of the CNS cellular population, while in rodents account for 5-12%, depending on brain region (Norden and Godbout, 2013). Microglial cells play a central role in neuroinflammation, being the major producers of pro-inflammatory mediators when activated. In the resting state, these cells present ramified extensions that constantly survey the surrounding microenvironment (Norden and Godbout, 2013). Microglia possess several families of pattern recognition receptors that can, upon binding of recognizable evolutionary conserved motifs of microbial and viral-derived molecules classified as pathogen-associated molecular patterns (PAMPs), activate downstream signalling cascades that culminate in microglial activation. Further, several of these receptors are also involved in the recognition of disparate molecules released from endogenous compartments or modified in structure, such as abnormal protein aggregates, that present damage-associated molecular patterns (DAMPs) (Landreth and Reed-Geaghan, 2009; Heneka et al., 2014).

Upon activation, microglia change morphologically into an amoeboid shape and display increased expression of specific cellular surface receptors and key enzymes, presenting increased phagocytic capabilities and secretion of inflammation-related molecules. Phenotypically, microglia can present M1 activation (or classical activation) or M2 activation (or alternative activation) (Lyman et al., 2013; Norden and Godbout, 2013). M1 activation is associated with an aggressive inflammatory response, leading to increased release of ROS and nitric oxide (NO), and production of pro-inflammatory mediators such as cytokines interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF-α), which are responsible for autocrine and paracrine inflammatory signalling in other glial cells and neurons. There is also increased production of chemokines such as IL-8 and

monocyte chemoattractant protein 1 (MCP1), which attract other microglia and peripheral macrophages to the injury site. On the other hand, M2 activation is classically stimulated by IL-4 and IL-13, being characterized by the repression of pro-inflammatory gene expression and increased production of neurotrophic factors and anti-inflammatory cytokines such as IL-10, transforming growth factor β (TGF-β), IL-4 and IL-13. This phenotype reduces inflammation, promotes phagocytic capacities and potentiates tissue repair (Mandrekar-Colucci and Landreth, 2010; Lyman et al., 2013; Meraz-Rios et al., 2013; Norden and Godbout, 2013).

Activated microglia are intimately associated with SPs in the brains of AD patients (Perlmutter et al., 1990). Activated microglia proliferate locally and contribute to the recruitment of more microglial cells through the expression of chemokines (Meraz-Rios et al., 2013). In AD pathology, initial microglial activation is thought to be regulated by direct binding of fAβ to several pattern recognition receptors, such as scavenger receptors and toll-like receptors (TLRs), inducing microglial activation and/or phagocytosis of bound Aβ (Wilkinson and El Khoury, 2012). These include the scavenger receptors cluster of differentiation (CD) 36, $\alpha_6\beta_1$-integrin, CD47 and scavenger receptor A-1 (SRA) multicomponent complex and the receptor for advanced glycation end-products (RAGE), as well as TLR2 and TLR4 (Bamberger et al., 2003; Wilkinson and El Khoury, 2012). Engagement of these receptors generate intracellular signalling cascades that culminate in the activation of transcription factors associated with the expression of pro-inflammatory genes, including nuclear factor κB (NF-κB), activator protein 1 (AP-1) and the signal transducer and activator of transcription 3 (STAT3), and/or receptor-mediated phagocytosis (Wilkinson and El Khoury, 2012).

Despite the generalized microglial activation around SPs, these cells are incapable of clearing these deposits by phagocytosis. Although several studies have determined that microglia can phagocyte Aβ *in vitro*, analysis of brain tissue from AD patients demonstrated that aggregated Aβ is not present in the lysosomal compartments of SPs-associated microglia (Frackowiak et al., 1992). Furthermore, there is evidence that microglia isolated from aged rodents are senescent, presenting reduced Aβ phagocytic capacity and increased pro-inflammatory responses (Norden and Godbout, 2013). Similar results are obtained with microglia isolated from non-demented and AD brains, with AD-derived microglia presenting wider range and levels

of pro-inflammatory signalling mediators (Lue et al., 2001). A recent study developed in APP/PS1 mice compared the expression levels of several Aβ receptors and Aβ-degrading enzymes in microglia isolated from old mice (Hickman et al., 2008). They observed decreased expression of SRA, CD36 and RAGE, along with reduced expression of the Aβ-degrading enzymes insulysin, neprilysin and matrix metalloproteinase 9 in microglia in older transgenic mice when compared to wild-type littermates. Importantly, this was not detected in younger APP/PS1 mice. Older microglia also presented increased expression of TNF-α and IL-1β. A similar study further characterized the phenotype of activated microglia of APP/PS1 mice at increasing ages, concluding that there is an age-dependent shift from M2 activation, a phagocytic state, to M1 activation, a classical cytotoxic phenotype (Jimenez et al., 2008). Interestingly, compelling data have been showing that basal neuroinflammatory conditions are also augmented in non-demented human aged brains, with microglia preferably adopting the M1 pro-inflammatory phenotype in the elderly (Norden and Godbout, 2013).

The development of a pro-inflammatory environment that negatively regulates microglial Aβ phagocytic activity indicates that loss of microglia Aβ clearance capacities is not only due to aging, but also to the chronic inflammatory milieu observed in AD pathology (Mandrekar-Colucci and Landreth, 2010).

These results strongly support the hypothesis that, during the early stages of AD, microglia may alleviate amyloid burden and provide support to injured neurons by adopting a M2 activation phenotype. However, with disease progression they shift towards the M1 activation state, losing Aβ clearance functions and further contributing to chronic neuroinflammation/neurodegeneration, with the formation of self-propelling cycles that potentiate AD-related pathologies.

2.2. Astrocytes

Astrocytes are the most abundant cells in the CNS, playing an essential role in brain homeostasis (Li et al., 2011). They are enrolled in innumerous functions, including regulation of neuronal metabolism, neurotransmitter synthesis and recycling, ion homeostasis and regulation of neural and synaptic transmission (Sofroniew and Vinters, 2010). They are also significant participators in the inflammatory response in

the CNS (Meraz-Rios et al., 2013). In AD, astrocytes are activated around SPs, leading to increased number, size and motility of astrocytes and ultimately resulting in the sequestration of SPs within an outer ring of reactive astrocytes (Li et al., 2011). When activated, astrocytes produce several pro-inflammatory cytokines and chemokines, along with NO and ROS production, contributing to neuronal damage (Hu et al., 1998; Johnstone et al., 1999; Li et al., 2011). Reactive astrocytes present high expression of intermediate filament proteins, including glial fibrillary acidic protein (GFAP) and vimentin (Pekny and Pekna, 2004; Wood-Kaczmar et al., 2009). Astrocytic activation also contributes to impaired neuronal activity. For example, reactive astrocytes reuptake the neurotransmitter glutamate inefficiently, which culminates in excitotoxicity to proximal neurons (Meraz-Rios et al., 2013). Studies *in vitro* have determined that astrocytes can be activated by exposure to Aβ aggregates, with subsequent production of pro-inflammatory cytokines, such as IL-1β, TNF-α, and inducible nitric oxide synthase (iNOS) with concomitant NO generation (White et al., 2005). It has been demonstrated that astrocytes express several receptors known to bind Aβ species and mediate Aβ-downstream pro-inflammatory signalling, such as receptor for advanced glycation end-products (RAGE) and TLRs (Trudler et al., 2010; Askarova et al., 2011). Importantly, a recent study has also reported Aβ-dependent astroglial activation *in vivo*, with injections of oligomeric $A\beta_{1-42}$ in the rat cortex (Perez et al., 2010; Carrero et al., 2012). These injections resulted in marked activation of NF-κB in astrocytes, with a significant up-regulation of TNF-α and IL-1β (Carrero et al., 2012). Although activated, astrocytes can phagocytose Aβ species upon binding to the receptors CD36 and CD47 *in vitro*. The importance of this ability to the progression of AD in patients or animal models is still to be determined (Jones et al., 2013).

Reactive astrocytes also express increased levels of S100B, a neurotrophic factor that induces APP expression in neurons, further contributing to Aβ generation (Li et al., 1998; Hamed et al., 2009). Apparently, S100B up-regulation depends on IL-1β levels (Liu et al., 2005a) involving S100B in a self-propagation cycle between Aβ and cytokine production, resulting in chronic neuroinflammation (Li et al., 2011).

Finally, it has been demonstrated that astrocytes stimulated with pro-inflammatory molecules usually overexpressed in AD, such as TNF-α, show increased APP and BACE1 levels, resulting in increased Aβ production (Blasko et al., 2000; Zhao

et al., 2011). Incubation of astrocytes with Aβo and fAβ dramatically increased APP and BACE1 levels through transcriptional mechanisms (Zhao et al., 2011). Therefore, although neurons are the main producers of Aβ, astrocytes outnumber neurons in a five-to-one ratio, implicating activated astrocytes not only in neuronal injury, but also as a source of Aβ during CNS inflammation.

2.3. Cytokines

Activated microglia and astrocytes secrete cytokines, which are soluble signalling proteins that influence the inflammatory response in several cell types. Characterization of the pro-inflammatory cytokine repertoire in AD patient brain tissue and cerebrospinal fluid revealed a generalized elevation of these molecules, namely IL-1β, IL-6, TNF-α and TGF-β (Blum-Degen et al., 1995; Licastro et al., 2000; Jiang et al., 2011; Meraz-Rios et al., 2013). These cytokines are highly produced in microglia and, to a minor extent, in astrocytes activated by Aβ aggregates (Tehranian et al., 2001; Meraz-Rios et al., 2013). Increased production of pro-inflammatory cytokines is also extensively described in AD mouse models (Sly et al., 2001; Tehranian et al., 2001; Patel et al., 2005). As such, several of these factors have been studied in AD contexts to evaluate their effect in AD progression.

2.3.1. TNF-α

TNF-α is a pro-inflammatory cytokine that can potentiate both microglial and astrocytic activation in several neuroinflammatory conditions, and especially in AD (Rossi et al., 2005; Rubio-Perez and Morillas-Ruiz, 2012). This cytokine has a dual role on neuronal survival and death by apoptosis, since TNF-α-downstream signalling mediated by TNF-α receptors (TNFR) 1 and 2 can either induce apoptosis by activation of caspase-8, or provide survival by activating NF-κB, depending on cellular context (Hohmann et al., 1990; Li et al., 2011). TNF-α can also inhibit neurogenesis, which might contribute to the cognitive decline observed in early stages of AD (Liu et al., 2005b). *In vitro*, TNF-α can potentiate γ-secretase activity, contributing to Aβ generation (Liao et al., 2004). Furthermore, TNF-α stimulated BACE1 expression, thus increasing amyloidogenic APP processing in cultured astrocytes overexpressing Swedish mutant APP (Yamamoto et al., 2007). These results might be explained by

the fact that TNF-α can activate NF-κB, which is a known inducer of BACE1 expression (Ly et al., 2013). TNF-α may also play an important role in regulating Aβ phagocytosis, since cultured microglia treated with this cytokine presented decreased expression of CD36 and SRA along with reduced Aβ uptake (Hickman et al., 2008).

Studies performed in a mouse model of chronic neuroinflammation reported increased TNF-α levels along with loss of cognitive function. Importantly, treatment with an analog of thalidomide that inhibits TNF-α synthesis, reduced hippocampus TNF-α levels to levels similar to control mice, while ameliorating cognitive deficits (Belarbi et al., 2012). Moreover, this same inhibitor has been transcribed as being therapeutic in an AD mouse model, attenuating amyloid deposition, decreasing gliosis and rescuing behavioral deficits (Tweedie et al., 2012). Other study administered an anti-TNF-α monoclonal antibody to APP/PS1 mice, which resulted in reduced Aβ accumulation and tau hyperphosphorylation (Shi et al., 2011). APP23 transgenic mice with genetic ablation of TNFR-1 also present decreased Aβ generation and attenuated cognitive deficits (He et al., 2007). However, ablation of both TNF-α receptors in 3xTgAD mice exacerbated Aβ pathology, while isolated microglia from this mouse model manifested decreased Aβ phagocytic activity (Montgomery et al., 2011). This indicates that TNF-α signalling may have both protective and detrimental roles in AD pathology, arguing against long-term pan-anti-TNF-α treatments in AD.

2.3.2. IL-1β

The IL-1 cytokine super family is basally expressed in the healthy CNS and includes IL-1α, IL-1β and IL-1 receptor antagonist. All CNS cells (neurons, microglia, astrocytes and oligodendrocytes) can synthesise IL-1β and express the respective signalling receptor (Fogal and Hewett, 2008). In physiological conditions, IL-1β is involved in the regulation of synaptic plasticity, contributing to LTP in the hippocampus (Balschun et al., 2003). However, higher levels of this cytokine, induced by either direct IL-1β injection or induction of an acute peripheral inflammatory response, impaired spatial memory in mice (Gibertini et al., 1995). IL-1β is a pro-inflammatory cytokine that sensitizes microglial cells and potently activates astrocytes (Carrero et al., 2012; Lyman et al., 2013). Upon binding to its receptor, IL-1β induces the activation of several signalling pathways, leading to increased production of IL-6 and IL-8 (Ridley et

al., 1997; Jung et al., 2002). IL-1β-dependent p38MAPK signalling promotes cyclooxygenase-2 expression, whose products further induce the expression of IL-6, thus generating a positive feedback loop (Lacroix and Rivest, 1998; Lyman et al., 2013). The p38MAPK is also a kinase known to phosphorylate tau in neurons, thus contributing to NFT formation (Li et al., 2003). In astrocytes, IL-1β increases the production of ROS and NO, further contributing to neuronal damage (Hu et al., 1998; Akama and Van Eldik, 2000). IL-1β can also contribute to Aβ production by increasing γ-secretase activity in a JNK-dependent manner (Liao et al., 2004).

Despite all the evidence implicating IL-1β in AD pathology, PS1Δ9 transgenic mice overexpressing IL-1β in the hippocampus presented decreased amyloid plaque formation with associated robust neuroinflammatory response (Shaftel et al., 2007). It was hypothesized that these effects might reflect an enhancement of microglial Aβ phagocytosis, further highlighting the importance of the subtle balance between several inflammatory factors in affecting microglial phenotypic activation. Of note, this mouse model presents elevated IL-1β levels since before amyloid deposition and not afterward, which complicates the interpretation of the endogenous role of this cytokine during AD progression.

2.3.3. IL-6

IL-6 is a potent pleiotropic cytokine capable of inducing a strong inflammatory response in glial cells. In the CNS, it can be secreted by microglia and, to a minor extent, by astrocytes (Lyman et al., 2013). IL-6 receptor is expressed not only in glial cells, but also in neurons. Studies in hippocampal tissue determined that IL-6 expression is essential for sustained up-regulation of TNF-α and IL-1β (Sparkman et al., 2006). IL-6 can up-regulate APP expression, further exacerbating Aβ production and generating another positive feedback loop (Ringheim et al., 1998). In neurons, IL-6 signalling can activate the CDK5/p35 complex, which contributes to tau hyperphosphorylation (Quintanilla et al., 2004; Kitazawa et al., 2005). IL-6 inhibits neurogenesis and neural differentiation in adult hippocampus (Liu et al., 2005b).

Overexpression of IL-6 in the brains of an AD mouse model attenuates Aβ production and deposition and induces glial activation with increased expression of phagocytic markers, suggesting an improved Aβ clearance capacity (Chakrabarty,

2010). Much like the IL-1β overexpressing AD mouse model, these results suggest that IL-6 up-regulation might promote Aβ clearance at earlier stages of AD pathology.

Overall, the chronic neuroinflammatory conditions detected in AD brains are clearly involved with neurotoxic mechanisms and the potentiation of Aβ and tau pathologies, which further drives inflammation (Fig. 5).

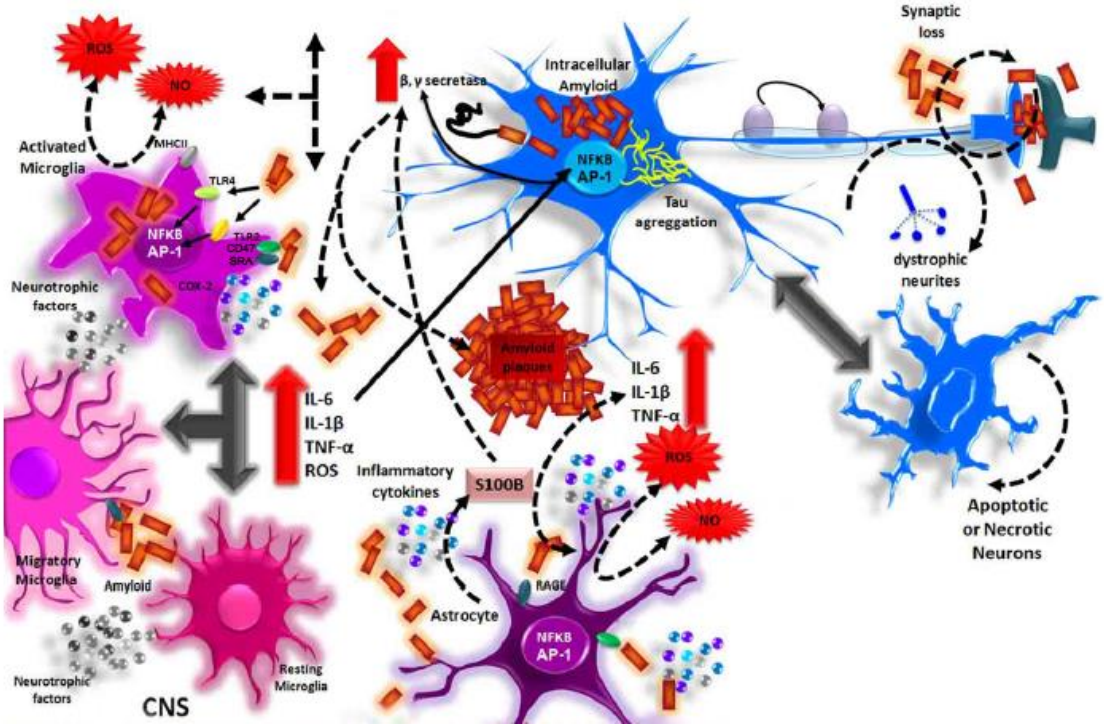


Fig. 5. Neuroinflammatory processes in Alzheimer's disease. Aggregated Aβ species act like DAMP-like molecules, being able to bind to several microglial receptors, including TLRs, RAGE, CD47 and SRA. Activation of these receptors then induces phagocytosis of bound Aβ and/or activation of transcription factors involved in the inflammatory response, which culminates in microglial activation and subsequent production of pro-inflammatory molecules, including oxidative species (ROS, RNS) and inflammatory cytokines (IL-1β, IL-6, TNF-α). Additionally, astrocytes are also important players in the inflammatory response by amplifying pro-inflammatory signalling, after becoming activated when exposed to Aβ and/or inflammatory molecules. In turn, a strong inflammatory environment contributes to deregulation of several neuronal mechanisms, leading to increased Aβ production, tau hyperphosphorylation and, ultimately, to neuronal dysfunction and death. Finally, elevated Aβ production further potentiates the inflammatory response, leading to the formation of a vicious cycle between both pathologies. Adapted from Meraz-Rios et al., 2013.

3. AD diagnosis and therapy

Recent guidelines for diagnostic criteria have been advanced that expand AD diagnosis into: (1) pre-symptomatic phase; (2) symptomatic, pre-dementia phase (MCI); and (3) dementia phase, based on clinical, neurological and psychiatric examinations, along with the usage of biomarkers that include biological fluid analyses and neuroimaging measures (Jack et al., 2011).

The blood and cerebrospinal fluid (CSF) provide numerous biomarkers for AD, but CSF biomarkers present higher consistency among studies. Promising CSF biomarkers include Aβ species and phosphorylated tau/total tau. Nevertheless, the levels of these proteins might vary in CSF depending on AD progression, which precludes a straightforward interpretation of the results (Reitz and Mayeux, 2014). Bioimaging techniques have also been employed in AD diagnosis, including magnetic resonance imaging (MRI), computed tomography (CT) and positron emission tomography (PET). Structural MRI and CT scans can detect cerebral atrophy, which can be related to AD depending on the affected regions (Reitz and Mayeux, 2014). Further, PET scanning has been applied for the detection of amyloid aggregates and NFTs, after the introduction of radiotracers into the blood stream that can reach the brain and bind these AD structures (Shoghi-Jadid et al., 2002; Reitz and Mayeux, 2014). PET can also be used to study cerebral metabolism by using a radiolabelled form of glucose. Importantly, cerebral metabolism is decreased in AD-affected brain regions (Landau et al., 2011). Despite this complexity and extensive overlapping of clinical features with other dementias, it is already possible to diagnose AD with some certainty. Nevertheless, definitive AD diagnosis can only be obtained post-mortem by microscopic examination of the cerebral cortex and subsequent identification of SPs and NFTs (Selkoe, 2001; Reitz and Mayeux, 2014). Unfortunately, available treatments for AD are only focused on the management of symptoms, without affecting disease progression. These include acetylcholinesterase inhibitors (donepezil, galanthamine, rivastigmine), which attempt to counteract impaired cholinergic function, and memantine, an antagonist of the NMDA subtype of glutamate receptors that prevent their overstimulation (Selkoe, 2001; Stone et al., 2011). Therefore, multiple studies have proposed novel therapeutic strategies targeting several of the pathways deregulated in AD.

Several of these strategies are focused on decreasing Aβ production/aggregation or enhancing the clearance of Aβ and aggregated tau. In this regard, posiphen, an inhibitor of β-secretase that precludes Aβ generation through the amyloidogenic pathway, is presently in clinical trials, with promising preliminary results (Maccecchini et al., 2012). Further, compounds described to decrease Aβ aggregation in AD mouse models, such as scyllo-inositol and melatonin, are also in clinical trials (Stone et al., 2011). Finally, Aβ-directed immunotherapy has been shown to reduce AD pathology in AD mouse models (Schenk et al., 1999; Lemere, 2013). However, active anti-Aβ immunization induces severe auto-immune reactions in AD patients, including meningoencephalitis, while passive immunotherapy with antibodies directed to Aβ, despite promising, have yielded no significant clinical benefits (Lemere, 2013).

Other options encompass antioxidant therapy with natural occurring antioxidants, such as vitamin E, C and B that appear to decrease AD risk, according to several longitudinal studies (Sano et al., 1997; Zandi et al., 2004; Stone et al., 2011). Interestingly, several synthetic antioxidants have been investigated in AD contexts, and the most promising results derive from compounds that target mitochondrial ROS production in AD *in vitro* and *in vivo* models (Siedlak et al., 2009; Stone et al., 2011).

Several epidemiologic studies have determined that subjects treated for long periods with nonsteroidal anti-inflammatory drugs (NSAIDs) present a significantly lower risk for incidence of AD (Broe et al., 2000; Cote et al., 2012). However, following studies show no clinical effect in patients diagnosed with mild-to-moderate AD (Reines et al., 2004; Stone et al., 2011). Controversial results were also described for prevention trials for several NSAIDs, including naproxen and celecoxib (Martin et al., 2008).

In conclusion, AD is a complex neurodegenerative disorder that depends on numerous interlocked mechanisms for the onset of its pathological features. Furthermore, AD mechanisms may also be generated and/or potentiated by deregulated processes observed in normal aging, thus increasing the number of players involved in this multifactorial pathology. Therefore, effective therapeutic intervention in AD will probably require multiple therapeutic agents directed toward several cellular and molecular deregulated targets implicated in AD.

4. Modeling AD *in vivo*

The need for AD animal models that mimic AD progression led to the establishment of a plethora of transgenic AD mouse models that carry diversified combinations of familial AD mutations in *APP* and/or *PS1*/*PS2* genes expressed from several promoters, which ultimately present Aβ deposition into SPs (Elder et al., 2010; Wirths and Bayer, 2010). Nevertheless, other AD-associated phenotypes are differentially observed among the available transgenic models, with none of them fully recapitulating the cognitive decline described in AD patients (Bryan et al., 2009; Webster et al., 2014). In fact, overexpression of APP and/or PS1 using artificial promoters poses several drawbacks, such as overproduction of APP and its cleavage fragments besides Aβ, which can also impact on AD-associated phenotypes (Born et al., 2014; Saito et al., 2014). Further, AD transgenic mice do not develop neurofibrillary tangle pathology, the second hallmark of AD, despite presenting tau hyperphosphorylation (Ribe et al., 2005). In this regard, mice carrying APP and PS1 transgenes, along with overexpression of mutant human tau, have been developed in order to recapitulate the features of tau pathology observed in AD patients, with some success (Wirths and Bayer, 2010; Ribe et al., 2005).

Nevertheless, double-transgenic mice carrying mutations associated with EOAD in *APP* and *PS1* genes are among the most successful models, developing Aβ plaques and a robust neuroinflammatory response towards these aggregates, along with loss of synaptic integrity and associated memory and cognitive impairments (Radde et al., 2006; Elder et al., 2010; Balducci and Forloni, 2011). However, most APP overexpressing transgenic mouse models of AD present limited and usually localized neuronal death several months after most AD-related features are already present, even in the presence of massive amyloid load (Elder et al., 2010; Wirths and Bayer, 2010; Rupp et al., 2011). These features contrast with AD patients in advanced stage of the disease, in which neuronal death can reach approximately 90% for layer II of the entorhinal cortex (Gomez-Isla et al., 1996). Several explanations for this general difference between AD patients and mouse models have been advanced. For example, neurons in the mouse brain can be more resistant to neurotoxic effects exerted by Aβ species derived from human APP overexpression, by presenting different coping mechanisms or different levels of neuronal Aβ targets; however,

cultured primary mouse neurons can still undergo massive apoptosis when exposed to Aβ (Watson and Fan, 2005). Further, overexpression of APP itself may be neurotrophic, since it has been demonstrated that several transgenic mice strains that overexpress APP with different mutations present higher cortical neuron size and number at early stages of disease, when compared to wild-type mice (Bondolfi et al., 2002; Oh et al., 2009). Moreover, Aβ produced from the overexpression of APP in mice brain lack most of the complex post-translational modifications, truncated forms and cross-linked features observed in the brains of AD patients, which might explain different neurotoxic effects (Kalback et al., 2002). Also, virtually no APP overexpressing mice develop NFTs, unless elevated tau levels are obtained artificially through transgenic approaches (Rupp et al., 2011). Interestingly, intraneuronal Aβ levels have been implicated in extensive neuronal loss in some APP/PS1 mouse models (Casas et al., 2004; Oakley et al., 2006).

Nevertheless, AD animal models remain instrumental for the elucidation of molecular pathways involved in AD and for the development of possible therapies to treat this devastating neurodegenerative disease.

5. Targeting the PI3K/Akt/GSK3 signalling pathway

The phosphatidylinositide 3-'OH kinase (PI3K)/Akt signalling pathway is implicated in the modulation of multiple cellular pathways involved in metabolism regulation and energy homeostasis (Peltier et al., 2007; O'Neill, 2013). There are many growth factors capable of activating PI3K, such as insulin and insulin-like growth factor 1 (IGF1). When activated, PI3K produces phosphatidylinositol-3,4,5-trisphosphate (PIP3), which in turn recruits the serine-threonine kinase Akt to the membrane. Here, Akt is phosphorylated at Thr308 by phosphoinositide-dependent protein kinase-1 (PDK-1), which allows for mTORC2-mediated phosphorylation of Akt at Ser473, fully activating this kinase (Fig. 6) (Beaulieu et al., 2009; O'Neill, 2013).

In the brain, the PI3K/Akt pathway is involved in the regulation of mechanisms of neuronal plasticity. During brain development, PI3K/Akt modulate proliferation, cell migration, axon guidance and establishment of neuronal polarity (Peltier et al., 2007; Akiyama and Kamiguchi, 2010). Further, PI3K/Akt downstream signalling induces the

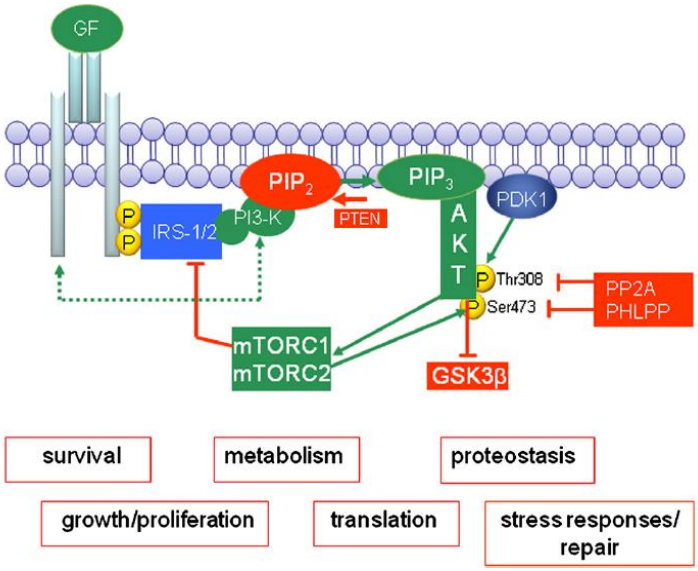


Fig. 6. Regulation of PI3K/Akt signalling. Binding of insulin, IGF1 or other growth factors activates their tyrosine kinase receptors by autophosphorylation, which then recruit IRS-1/2, thereby activating PI3K. PI3K produces PIP3, which recruits Akt to the membrane, allowing for the interaction with PDK1 and subsequent phosphorylation of Akt at Thr308. This allows for mTORC2-mediated phosphorylation of Akt at Ser473, which fully activates this kinase. Akt can then phosphorylate a broad range of substrates involved in cellular function, including GSK3. Termination of PI3K/Akt signal transduction depends on dephosphorylation of PIP3 by phosphatase and tensin homolog (PTEN), dephosphorylation of Akt by PP2A and PH domain leucine-rich repeat protein phosphatases (PHLPPs), and negative feedback inhibition via mTOR/mTORC1. Adapted from O'Neill, 2013.

activation of the cAMP response element-binding (CREB) transcription factor, which in turn promotes proliferation of hippocampal neuronal cells (Peltier et al, 2007). In the adult brain, PI3K/Akt signalling increases the survival rate of new hippocampal granule cells in the dentate gyrus, thus increasing synaptic plasticity (Bruel-Jungerman et al., 2009).

In fact, activation of PI3K/Akt signalling is crucial for hippocampal expression of late-phase long-term potentiation (LTP), a form of synaptic plasticity closely related with learning and memory (Horwood et al., 2006; Karpova et al., 2006). Interestingly, insulin receptors appear to be directly implicated in learning and memory by upregulating synaptic function in the hippocampus, which depends on PI3K/Akt activation (Zhao et al., 1999). Akt expression is also necessary for neuronal survival during normal and stress-inducing conditions, especially in those involving oxidative

stress (Crowder and Freeman, 1998; Kang et al., 2003; Murata et al., 2011). Finally, the PI3K/Akt/mammalian target of rapamycin kinase (mTOR) pathways increases both presynaptic and postsynaptic protein synthesis, an important mechanism for the maintenance of enhanced synaptic strength (Je et al., 2011).

Importantly, Akt can phosphorylate GSK3α/β at serine 21/9, which strongly inhibits the activity of this multifaceted enzyme (Beaulieu et al., 2009). GSK3 is a partially constitutively active serine/threonine kinase ubiquitously expressed in all mammals. GSK3 is generated from two distinct genes as two highly homologous isoforms, GSK3α and GSK3β (Woodgett, 1991; Soutar et al., 2010). Although both isoforms exert similar functions, they are differentially expressed among cell types, being GSK3β preferentially expressed in the nervous tissue (Soutar et al., 2010). Initially identified by its ability to phosphorylate glycogen synthase, GSK3 is now known to be a multifunctional enzyme targeted by many signalling pathways, being able to phosphorylate more than 50 substrates (Jope and Johnson, 2004; Sutherland, 2011). Several of these targets are transcription factors, further highlighting the fundamental role of GSK3 in the regulation of cellular responses (Jope and Johnson, 2004). Due to its multiple targets, GSK3 activity is tightly regulated. One main upstream regulatory mechanism is inhibition of GSK3 by direct phosphorylation of Ser21 in GSKα, and Ser9 in GSK3β (Sutherland et al., 1993; Jope and Johnson, 2004). Several kinases can phosphorylate this residue, including protein kinase A (Votypka et al.), protein kinase B (PKB)/Akt and protein kinase C (PKC) (Jope and Johnson, 2004). On the other hand, optimal GSK3 activity is achieved by phosphorylation of Tyr279 in GSKα, and Tyr216 in GSK3β (Lochhead et al., 2006). Other regulatory mechanisms are mostly engaged in the fine-tuning of GSK3 selectivity for specific substrates. Downstream mechanisms include pre-phosphorylation (priming) of the substrates at 4-5 residues from GSK3 phosphorylation site. Most substrates must be primed to be phosphorylated by GSK3, which implicate coordinated activity between the priming kinase and GSK3 (Dajani et al., 2001). GSK3 is also contained in multi-protein complexes that regulate substrate accessibility, as exemplified by the substrate β-catenin in the Wnt pathway (Frame and Cohen, 2001). GSK3 is also translocated to the nucleus and mitochondria, and regulation of its subcellular location or its inhibitory-phosphorylation state in these

cellular compartments further restricts substrate accessibility (Jope and Johnson, 2004).

5.1. PI3K/Akt/GSK3 deregulation in AD

The PI3K/Akt/GSK3 axis is consistently described as being deregulated in AD (Ryder et al., 2004; Lee et al., 2009; de la Monte, 2012; Durairajan et al., 2012). Similarly, brain insulin resistance is widely detected in the brains of AD patients and mouse models, leading to reduced responses of the insulin signalling pathway mediated by the insulin receptor (IR), insulin receptor substrate-1 and -2 (IRS1,2) and PI3K/Akt pathway (Liu et al., 2011; de la Monte, 2012; Talbot et al., 2012). Interestingly, Aβ-induced cellular stress has been correlated with activation of JNK, which can phosphorylate and abolish the activity of IRS-1,2 leading to impaired insulin/PI3K/Akt signalling (Bomfim et al., 2012; Jia et al; 2013). Ultimately, this impacts on synaptic plasticity, neuron metabolism, neurodegeneration and cognitive decline (de la Monte, 2012; Liu et al., 2011). *In vitro*, Aβ-induced apoptosis is also readily inhibited by the activation of the PI3K/Akt pathway (Sola et al., 2003; Shi et al., 2010; Kong et al., 2013).

Conversely, several reports have detected abnormal upregulation of PI3K/Akt signalling pathway in AD brains, which can be involved in the development of insulin resistance through feedback mechanisms (Talbot et al., 2012). Oral administration of the PI3K inhibitor wortmannin resulted in reduced Aβ deposition in the Tg2576 transgenic mouse model of AD (Haugabook et al., 2001), and PI3K inhibition in neuroblastoma cells overexpressing APP resulted in decreased Aβ production and secretion, along with APP accumulation inside the cells, probably due to limited vesicular trafficking (Petanceska and Gandy, 1999). Increased mTOR activity, a downstream target of PI3K/Akt, also appears to be associated with AD progression. In this regard, mTOR can upregulate both tau and APP (Westmark and Malter, 2007; Morita and Sobue, 2009). Further, mTOR negatively modulates autophagy and the clearance of protein aggregates, which possibly contributes to neurodegeneration (Heras-Sandoval et al., 2014).

Therefore, the contribution of the PI3K/Akt pathway to AD is still a matter of intense debate and is far from clear. One possible explanation is that the PI3K family

comprises different PI3K isoforms that are differentially expressed among the CNS (Rodgers and Theibert, 2002). The differential expression of these various isoforms, allied to their subcellular location and metabolic cellular context (Hawkins et al., 2006) can possibly justify the divergent opinions toward the role of PI3K/Akt signalling in AD. Further work is thus needed to elucidate the complex mechanisms linking insulin resistance, PI3K/Akt deregulation and AD progression.

On the other hand, GSK3 hyperactivity has been extensively correlated with AD pathology. The brains of AD patients present increased GSK3 activity in affected brain regions, a feature widely mimicked *in vivo* by AD mice, and *in vitro* by cell cultures harbouring familial AD mutations or exposed to Aβ (Ryder et al., 2004; Lee et al., 2009; Jimenez et al., 2011; Durairajan et al., 2012).

In this regard, GSK3β is known to be the main kinase responsible for tau phosphorylation at multiple sites, and deregulation of this enzyme is consequently highly involved in tau hyperphosphorylation and subsequent formation of NFTs (Ishiguro et al., 1993; Takashima et al., 1998). Moreover, GSK3β can also phosphorylate APP, which increases APP endoproteolysis through the amyloidogenic pathway (Aplin et al., 1996; Lee et al., 2003). GSK3-dependent phosphorylation of APP (p-APP) at the cytoplasmic residue Thr668 targets the protein for fast axonal transport to nerve terminals. Axonal transport increases the co-localization of p-APP with β- and γ-secretases within axonal or presynaptic vesicles, which culminates in enhanced Aβ generation (Lee et al., 2003; Lee et al., 2005; Durairajan et al., 2012). Interestingly, specific GSK3β inhibition resulted in decreased BACE1 expression in an AD mouse model, with a concomitant decrease in the amyloidogenic processing of APP (Ly et al., 2013). These results implicate GSK3 in both APP phosphorylation and elevated BACE1 expression, two processes involved in increased amyloidogenic processing of APP. GSK3β overexpression also impairs learning and memory in mice (Zhu et al., 2007). Moreover, GSK3 can activate intrinsic pro-apoptotic pathways in injured neurons (Fig. 7) (Mines et al., 2011). Increased GSK3β activity is also associated with decreased β-catenin levels in neural progenitor cells, impairing neurogenesis (Kitazawa et al., 2011).

Finally, inhibition of GSK3 *in vivo* has already been proven to ameliorate all major AD-associated pathologies (Sereno et al., 2009; Durairajan et al., 2012).

Overall, GSK3 is implicated in all major pathological mechanisms involved in AD, highlighting the modulation of GSK3 activity as a promising therapeutic target to treat AD.

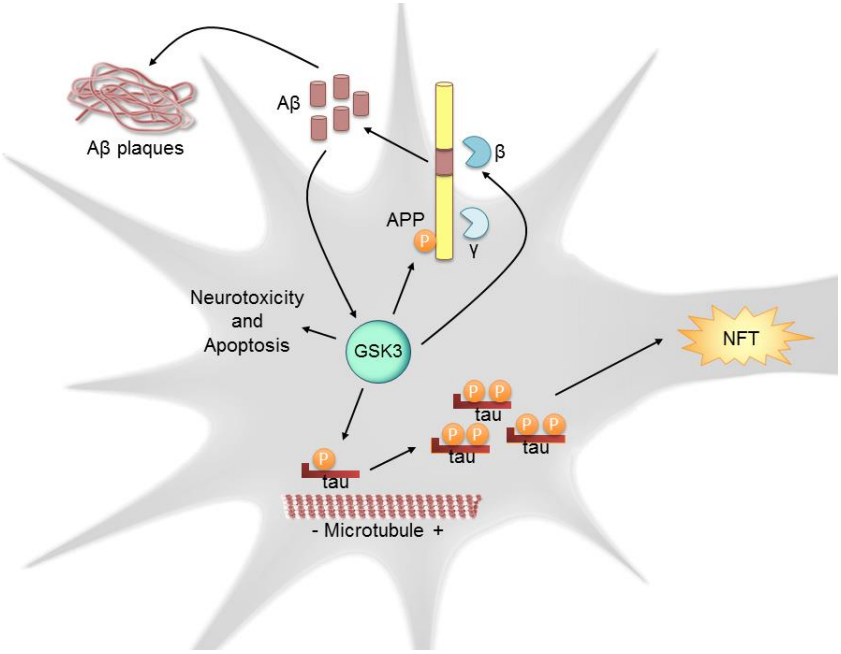


Fig. 7. Proposed mechanisms linking neuronal GSK3 deregulation and AD progression. In neurons, deregulated GSK3 activated is highly involved in tau hyperphosphorylation and subsequent formation of NFTs, a pathological hallmark of AD. GSK3-dependent phosphorylation of APP is also believed to increase Aβ production. Interestingly, GSK3 may also increase BACE1 expression, further elevating Aβ generation. Moreover, GSK3 hyperactivity is linked to disrupted neuronal function and induction of apoptotic pathways during stress conditions. Adapted from Jope et al., 2004.

5.1. GSK3 in neuroinflammation

GSK3 is an important mediator of innate and adaptive immune responses (Beurel et al., 2010). Interestingly, GSK3 was recently identified as playing a key role in pathways involved in glial activation, which is relevant to AD (Koistinaho et al., 2011). Inhibition of GSK3 significantly decreases microglial migration in both *in vitro* cultures and hippocampal organotypic slices (Yuskaitis and Jope, 2009). Furthermore, GSK3 inhibition also results in decreased microglial inflammatory activation, reducing production of IL-6 and iNOS and protecting against inflammation-induced neurotoxicity (Yuskaitis and Jope, 2009).

GSK3 can regulate the expression of several inflammatory mediators, usually promoting the expression of pro-inflammatory molecules, while decreasing anti-inflammatory factors (Martin et al., 2005; Beurel et al., 2010; Koistinaho et al., 2011). It has been established that this effect depends on the regulatory role of GSK3 on the activation of multiple transcription factors and interaction with signalling pathways, including NF-κB, STAT3 and the JNK pathway (Steinbrecher et al., 2005; Wang et al., 2010). In microglia, GSK3-dependent activation of NF-κB results in increased expression of iNOS and pro-inflammatory cytokines and chemokines, such as TNFα, and IL-6 (Fig. 8) (Yuskaitis and Jope, 2009; Wang et al., 2010; Koistinaho et al., 2011).

Further, inactivation of GSK3 increases inflammatory tolerance in astrocytes upon repeated inflammatory stimuli, indicating that inhibition of GSK3 might be beneficial during chronic neuroinflammatory conditions by decreasing sustained astroglial inflammatory response (Beurel and Jope, 2010). GSK3 has also been implicated in the activation of the transcription factor CCAAT/Enhancer-binding protein Delta (CEBPD) by direct phosphorylation, which appears to alter the pool of inflammatory molecules secreted by astrocytes (Ko et al., 2014).

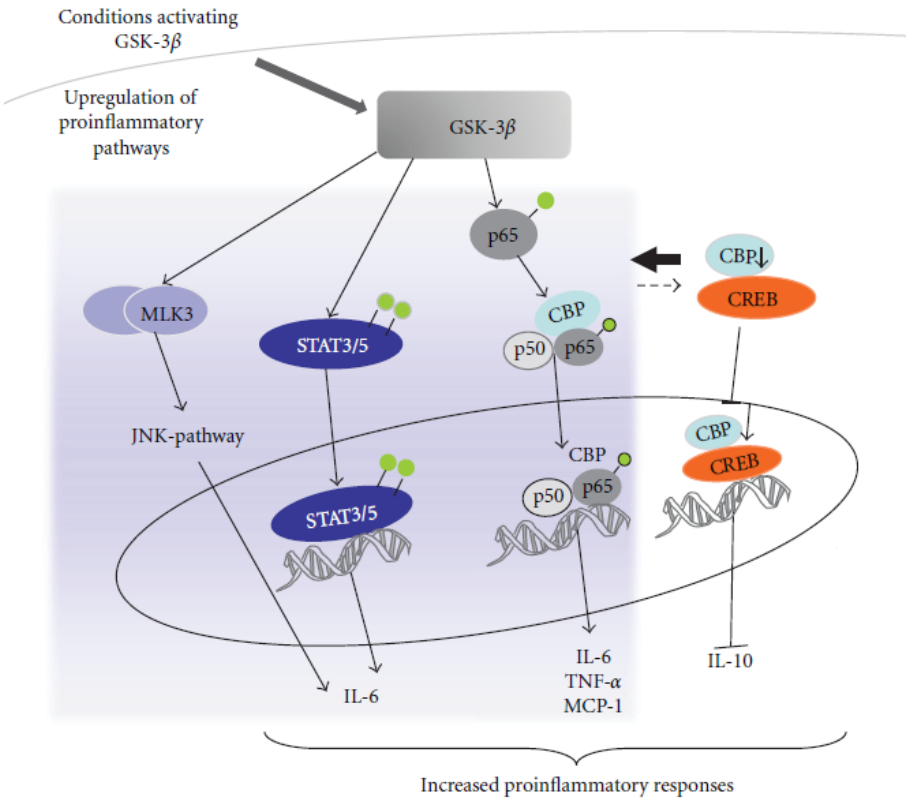


Fig. 8. GSK3β upregulates pro-inflammatory pathways in microglia. It has been demonstrated that GSK3β can directly activate transcription factors involved in the expression

of pro-inflammatory genes by phosphorylation, such as STAT3 and the p65 subunit of NF-κB. In the nucleus, p65 binds the co-activator of transcription CREB-binding protein (CBP) making it is less available to bind CREB, and upregulate expression of anti-inflammatory IL-10. Further, GSK3 can also upregulate IL-6 expression by mixed lineage kinase 3 (MLK3)-dependent activation of JNK pathways. Adapted from Koistinaho et al., 2011.

6. Using bile acids as a therapeutic approach

Bile acids are a class of acidic sterols that present a cyclopentanoperhydrophenanthrene nucleus (ABCD-ring) usually derived from a C_{24}-skeleton with a terminal carboxylic acid group, constituting the main type of molecules secreted in the bile. Bile acids constitute the main type of molecules secreted in bile, being synthesized in the liver from neutral sterols through complex series of chemical reactions catalyzed by multiple enzymes that are involved in the cholesterol metabolic pathway (Russell and Setchell, 1992). Interestingly, the intermediates and end-products of bile acid pathways modulate the expression of genes involved in the synthesis of cholesterol, fatty acids and bile acids, mainly by interacting with DNA-binding nuclear receptors (Repa and Mangelsdorf, 1999). The primary bile acids derived from bile acid biosynthesis are usually conjugated with glycine or taurine, being then secreted via the bile ducts into the small intestine. There, bile acids act mainly as detergents to emulsify dietary lipids, due to their amphipathic structure that allows them to be water-soluble and capable of inserting themselves into biological membranes. After uptake of emulsified nutrients, bile acids are re-absorbed to the liver via the portal vein, and are re-directed to the gallbladder for storage until the next feeding cycle (Russell and Setchell, 1992). Interestingly, the primary bile acid pool that enters the small intestine can suffer biotransformation catalyzed by intestinal bacteria, yielding secondary and terciary bile acids, such as ursodeoxycholic acid (UDCA) (Hagey et al., 1993).

Modifications of bile acids lead to altered physicochemical properties, either increasing or decreasing their hydrophobicity. Importantly, hydrophobic bile acids can act as cytotoxic molecules not only due to destabilization of biological membranes, but also as activators of extrinsic and intrinsic death pathways within the cell. On the other hand, hydrophilic bile acids such as UDCA are cytoprotective in several cellular contexts (Amaral et al., 2009).

UDCA is a hydrophilic bile acid increasingly used for the treatment of patients with hepatobiliary disorders, being widely considered as the first choice therapy for chronic cholestatic diseases (Roma et al., 2011). Interestingly, the cytoprotective properties of UDCA derive from its ability to abrogate apoptosis, although the molecular mechanisms engaged in this response are still not entirely unraveled. It is known that it involves stabilization of mitochondrial membranes, decreased Bax translocation into the mitochondria and reduced opening of the permeability transition pore, which further restricts cytochrome *c* release and subsequent caspase activation and substrate cleavage (Rodrigues et al., 1998a; Rodrigues et al., 1998b; Rodrigues et al., 1999). Further, UDCA has been demonstrated to strongly modulate the expression of several genes involved in apoptosis, cell cycle regulation and proliferation in hepatocytes (Castro et al., 2005).

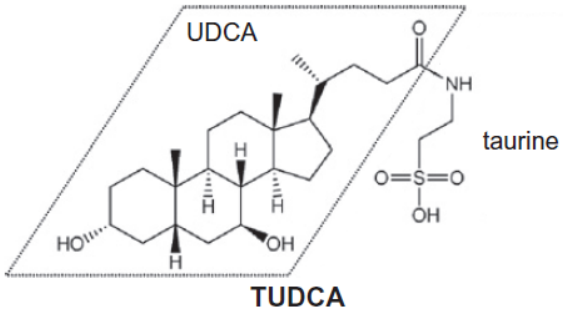


Fig. 10. Chemical structure of UDCA, here presented as the taurine conjugate form of UDCA. Adapted from Berger and Haller, 2011.

Tauroursodeoxycholic acid (TUDCA) is an endogenous hydrophilic bile acid that derives from the conjugation of UDCA with taurine (Fig. 10) (Amaral et al., 2009).

Importantly, TUDCA presents strong anti-apoptotic properties in multiple cellular contexts, including several experimental models of neurodegeneration. TUDCA is also a potent inhibitor of the ER stress-mediated pathways, reducing calcium efflux and activation of caspase-12 (Xie et al., 2002; Zhang et al., 2014). Importantly, TUDCA inhibits multiple Aβ-induced apoptotic mechanisms. A striking feature of TUDCA is its ability to act as a mitochondrial membrane stabilizer, abrogating membrane swelling, lipid peroxidation and cytochrome *c* release in neuronal cells and isolated rat mitochondria exposed to Aβ species (Rodrigues et al., 2000; Rodrigues et al., 2001). Further, TUDCA can also prevent Aβ-induced apoptosis by reducing JNK-dependent

activation of caspase-2, and interfering with p53-dependent pathways (Ramalho et al., 2004; Ramalho et al., 2006; Viana et al., 2010).

Curiously, bile acids are chemically similar to steroid hormones, thus allowing their interactions with nuclear steroid receptors (NSR) (Sola et al., 2006a). In fact, TUDCA can physically interact with the NSR mineralocorticoid receptor (MR), thus counter-acting the reduced transactivation of this receptor after Aβ exposure and subsequently downregulating Aβ-dependent apoptosis (Sola et al., 2006b). Moreover, TUDCA decreases Aβ and glutamate-induced mitochondrial dependent-apoptosis in primary rat cortical neurons by activating the pro-survival PI3K/Akt signalling cascade, which leads to Bad phosphorylation and subsequent reduction of Bad translocation into the mitochondria (Fig. 9) (Sola et al., 2003; Castro et al., 2004). Interestingly, TUDCA has also been reported to inactivate GSK3β and reduce inflammation in liver tissue after partial hepatectomy, suggesting that TUDCA may target the Akt/GSK3 pathway in several cell types (Ben Mosbah et al., 2010).

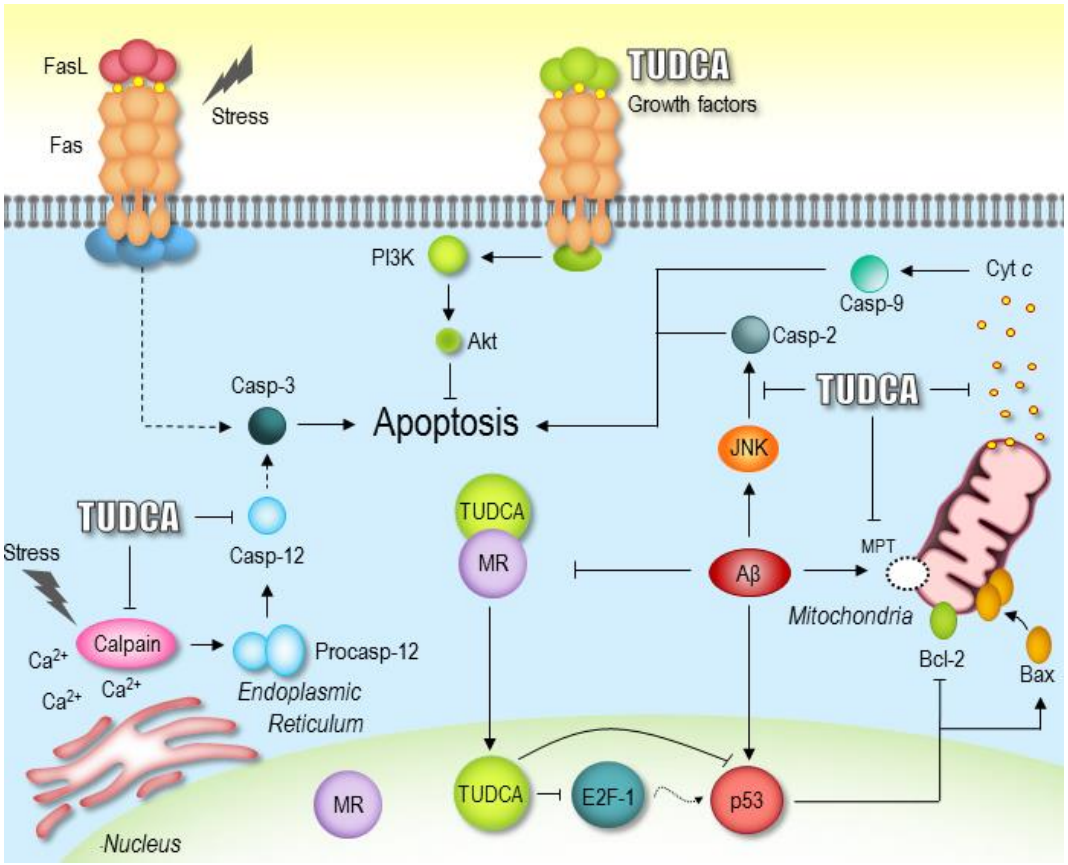


Fig. 9. Anti-apoptotic mechanisms elicited by TUDCA. TUDCA potently inhibits ER stress, thus precluding calcium leakage and subsequent caspase-12 activation. TUDCA can also protect from Aβ-induced apoptosis by modulating multiple pathways. In this regard, TUDCA

inhibits the apoptotic mitochondrial pathway by inhibiting Bax translocation, mitochondrial membrane permeabilization, cytochrome c release, and caspase-activation. TUDCA can also interact with the mineralocorticoid receptor (MR), forming a functional complex that undergo nuclear translocation. There, TUDCA downregulates p53 activity, probably through the transcription factor E2F-1, which further prevents apoptosis. TUDCA can also prevent JNK-dependent caspase-2 activation, while eliciting the activation of pro-survival pathways, such as PI3K/Akt. Adapted from Amaral et al., 2009.

Importantly, TUDCA enters the systemic circulation by oral administration and is able to cross the blood-brain barrier, greatly increasing the brain bioavailability of this bile acid (Keene et al., 2002). In this respect, the neuroprotective effects of TUDCA have been demonstrated in several animal models of neurodegenerative diseases, including Alzheimer's, Huntington's (Keene et al., 2001; Keene et al., 2002) and Parkinson's diseases (Duan et al., 2002; Castro-Caldas et al., 2012), and ischemic and hemorrhagic stroke (Rodrigues et al., 2002; Rodrigues et al., 2003). Regarding AD, TUDCA presents therapeutic efficacy in APP/PS1 double-transgenic mice fed with a diet containing 0.4% TUDCA for 6 months (Nunes et al., 2012). Notably, TUDCA treatment significantly decreased Aβ levels and deposition in mice brain as well as neuroinflammation, which was markedly correlated with amelioration of memory deficits (Lo et al., 2013; Nunes et al., 2012). Further, TUDCA reduced the loss of the postsynaptic marker postsynaptic density-95 (PSD-95) and the decrease in spontaneous miniature excitatory postsynaptic currents (mEPSCs) frequency, while increasing dendritic spines density in primary rat cortical cultures exposed to Aβ. More importantly, TUDCA treatment of APP/PS1 mice abrogated the reduction in PSD-95 reactivity in the hippocampus (Ramalho et al., 2013).

Finally, TUDCA appears to possess intrinsic anti-inflammatory properties. In this regard, TUDCA has been shown to decrease glial activation and microglial migration in acute neuroinflammation models both *in vivo* and *in vitro* (Yanguas-Casas et al., 2014). Other studies also showed the anti-inflammatory properties of bile acids similar to TUDCA, such as UDCA and glycoursodeoxycholic acid (GUDCA), in astrocytes and microglia exposed to pro-inflammatory stimuli, including Aβ (Joo et al., 2003; Fernandes et al., 2007).

7. Objectives

Given the promising results of TUDCA treatment before the onset of amyloid pathology in an AD transgenic mouse model, we hypothesized that administration of TUDCA after disease onset is beneficial and ameliorates Aβ pathology. TUDCA has already been demonstrated to activate the PI3K/Akt pathway, highlighting this pathway as a possible relevant target of TUDCA in an animal model that overproduces Aβ. Further, Akt negatively regulates GSK3, an enzyme widely characterized as hyperactivated in AD and involved in all its major aspects. Finally, TUDCA presents anti-inflammatory properties

The main objectives of this study were:

- To evaluate whether TUDCA modulates Aβ deposition and production;
- To determine the effect of TUDCA on GSK3 activity levels and its downstream targets;
- To characterize the protective role of TUDCA in AD-mediated neuroinflammatory response.

Due to the lack of appropriate therapies to treat AD, TUDCA stands as a promising therapeutic strategy to treat this disease, possibly by acting on multiple therapeutic targets.

II. MATERIALS AND METHODS

Transgenic mice and treatment

APP/PS1 double-transgenic mice, maintained on a C57BL/6J genetic background, coexpress KM670/671NL “Swedish” mutated human APP695 and L166P mutated human PS1 under the regulatory control of a murine Thy-1 minigene promoter, which is a neuron-specific promoter that restricts transgene expression to the postnatal period (Radde et al., 2006). The Swedish double APP mutation leads to early-onset familial AD, mainly by increasing APP affinity for β-secretase, which results in elevated amyloidogenic processing of APP and subsequent higher Aβ production (Mullan et al., 1992;Haas et al., 2012). Since this mutation strongly enhances Aβ production, it is widely used for the generation of mouse models displaying Aβ pathology (Balducci and Forloni, 2011). The Leucine to Proline mutation at position 166 of PS1 is one of the most aggressive familial AD mutation described so far, since its presence accelerates disease onset to as early as 24 years of age (Moehlmann, 2002; Bentahir et al., 2006). The PS1-L166P mutation induces the highest $A\beta_{42}$ to $A\beta_{40}$ ratio in transfected cells among several other PS1 mutations (Moehlmann et al., 2002).

Mice were genotyped by PCR analysis of tail DNA. All animals were housed in standard cages with *ad libitum* access to food and water in a temperature-controlled environment with a 12h light/dark cycle. Male APP/PS1 mice and wild-type (wt) littermates were randomly assigned to four groups: TUDCA-treated and untreated (control) wt mice, and TUDCA-treated and untreated (control) APP/PS1 mice. At 7 months of age, animals were injected with either TUDCA (500 mg/kg of body weight) or vehicle every 3 days for 3 months (Fig. 1).

The dosage of TUDCA used was calculated based on previous studies developed in rodents, including APP/PS1 mice (Keene et al., 2002; Nunes et al., 2012). Animals with 7 months of age were used to evaluate the protective effects of TUDCA after the onset of amyloid pathology, since amyloid deposition in APP/PS1 mice starts at 6 weeks of age in the neocortex while, in the hippocampus, deposition starts at 2-3 months in the dentate gyrus and 3-4 months in the Cornu Ammonis 1 (CA1) (Radde et al., 2006).

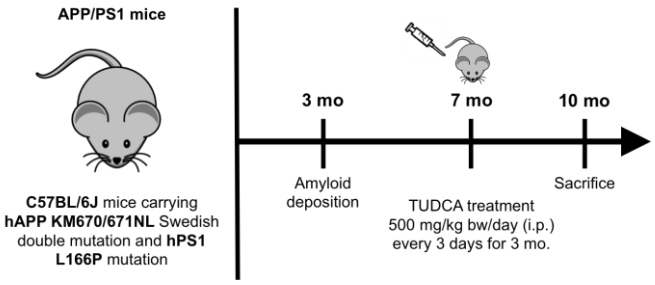


Fig. 1. Schematic timeline for TUDCA treatment in wt and APP/PS1 mice. Important time points are shown: at 2 months, APP/PS1 mice start presenting Aβ deposition in both neocortex and hippocampus; at 7 months of age, when amyloid pathology is firmly established, intraperitoneal (i.p.) injections containing TUDCA were administrated every 3 days for 3 months; at 10 months, all animal were tested for special memory and then sacrificed.

Morris Water Maze

Spatial learning was evaluated in the Morris water maze as previously described (Goddyn et al., 2006; Lo et al., 2013). Briefly, mice were trained in a total of 10 days (specifically in 2 series of 5 training days, with 2 days of rest between each series) to find a submerged platform. Four trials starting from four different starting positions were performed each day with a trial interval of 30 min. When mice failed to find the hidden platform within 2 min, they were guided to the platform and were left there for 15 s, before being returned to their cages. Latency to find the hidden platform was recorded with Ethovision (Noldus Bv, Wageningen, The Netherlands). To evaluate retention memory, probe trials were introduced on days 6 and 11. During these probe trials, the platform was removed and the swimming path was recorded during 100 s. Time spent in each quadrant was measured.

Immunohistochemistry

Saline-perfused brains were excised and one hemisphere was snap frozen for protein extraction. The other hemisphere was fixed in 4% paraformaldehyde for 48 h and stored in 30% sucrose/phosphate buffered saline (PBS) at 4ºC. The treated hemispheres were further dehydrated and embedded in paraffin. Sequential coronal brain sections (4-µm thick) were obtained and mounted on SuperFrost-Plus glass slides (Thermo Scientific, Rockford, IL, USA). For immunostaining, brain sections were

deparaffined, rehydrated and antigen retrieval was performed by boiling the sections for 20 min in 10 mM citrate buffer, pH 6.

The sections were then blocked for 1 h in Tris buffered saline (TBS) containing 10% (v/v) normal donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) and 0.1 % (v/v) Triton X-100 (Sigma-Aldrich) and subsequently incubated in appropriately diluted primary antibodies overnight at 4ºC. After washing with TBS/0.025% Tween20, the primary antibodies were developed with diluted (1:200) Alexa Fluor 568 (anti-mouse) or Alexa Fluor 594 (anti-rabbit) conjugated secondary antibodies (Invitrogen) for 2 h at room temperature. After rinsing, the sections were counterstained with Hoechst 33258 (Sigma-Aldrich) and mounted on Fluoromount (Sigma-Aldrich). The following primary antibodies were used: Aβ deposits were stained with a mouse monoclonal anti-Aβ antibody (6E10; Covance; 1:1,000); astrocytes were stained with a mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (GA5; Millipore Corporation, Temecula, CA, USA; 1:200); microglia were stained with a rabbit polyclonal anti-Iba-I antibody (Wako Pure Chemicals, Richmond, VA, USA; 1:100); p-APP was stained with a rabbit polyclonal anti-p-APP (Thr668) antibody (Cell Signalling; 1:200); APP was stained with a rabbit polyclonal anti-APP, C-terminal antibody (Sigma-Aldrich; 1:200); p-tau was stained with a rabbit polyclonal anti-p-tau (Ser396) antibody (101815; Santa Cruz Biotechnology; 1:50); presynaptic terminals were stained with a mouse monoclonal anti-synaptophysin (SYN) antibody (SY38; Millipore; 1:100).

Histochemistry

After deparaffinization and rehydration, sections were stained with Thioflavin T, a highly sensitive marker of Aβ deposits. Staining was performed with freshly filtered 0.05% Thioflavin T (Sigma-Aldrich) solution in PBS for 8 min at room temperature (Nunes et al., 2012).

Image Analysis

All images were captured using an Axioskop fluorescence microscope (Carl Zeiss GmbH, Hamburg, Germany). At least eight images per hippocampal and cortical regions were acquired for each animal and converted to gray scale with an 8-bit format.

Semiquantitative analysis of GFAP, Iba-I, SYN and p-tau were performed with ImageJ 1.46r software (National Institute of Health, Bethesda, MD, USA). The background of each set of images was subtracted and a threshold optical density was determined and held constant. Mean gray values (MGV) obtained for GFAP, Iba-I, SYN and p-tau immunostaining in the four mice groups were normalized to the MGV units of control wt mice and are presented as percentage of wt control mice. The number of Aβ-plaques stained with Thioflavin T or 6E10 antibody were counted and presented as plaque number per square millimeter for both hippocampus and frontal cortex. Measurement of amyloid (Thioflavin) and Aβ (6E10) burden were also performed in thresholded images by applying an unbiased computer-assisted image analyzer available in the ImageJ software, which was used to quantify the areas occupied by positive staining in the regions of interest. Amyloid and Aβ burden was then calculated by normalizing the reactive area to the total area of the regions of interest, and is presented as percentage of the total area.

Real-Time PCR

Dissected hippocampus and frontal cortex were homogenized in TRIzol™ (Invitrogen) using a motor-driven Bio-vortexer (No1083; Biospec Products, Bartlesfield, OK). After homogenization, total RNA was isolated according to the manufacturer's protocol. Briefly, after homogenization in TRIzol, samples were maintained at room temperature for 5 min, followed by chloroform addition. Samples were then vortexed and incubated at room temperature for 3 min. Afterwards, samples were centrifuged at 12000 g for 15 min at 4ºC, to separate the aqueous phase (containing RNA), interphase (DNA) and organic phase (proteins). The aqueous phase was isolated to a new tube and isopropanol was added to precipitate RNA, while the organic phase – TRIzol-chloroform fractions – was further processed for protein extraction (see below). Samples were incubated for 2 h at -20ºC, followed by centrifugation at 12,000 g for 10 min at 4ºC. The RNA pellet was then washed with 75% ethanol, centrifuged at 7,500 g for 5min at 4ºC. Finally, the RNA pellet was allowed to dry for 5 min and further dissolved in autoclaved miliQ water. Isolated RNA was quantified using a Qubit™ 2.0 fluorometer (Invitrogen). Total RNA was converted into cDNA using Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative Real-time PCR (qRT-PCR) analyses were performed in the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), using SYBR Green

PCR master mix (Fermentas International Inc., Glen Burnie, Maryland, USA). The expression levels of the genes of interest relative to the housekeeping gene Hypoxanthine-guanine Phosphoribosyltransferase (HPRT) were calculated using the $\Delta\Delta C_t$ method. Control wt mice were used as the calibrator and the relative changes in gene expression were calculated according to the formula $2^{-\Delta\Delta Ct}$. Primer sequences are presented in Table 1.

Table 1. Primer sequences used to amplify indicated mouse cDNAs

	Sense primer (5'-3')	**Antisense primer (5'-3')**
HPRT	CAGTCCCAGCGTCGTGATTA	TGGCCTCCCATCTCCTTCAT
BACE1	TCCTTCCTCAGCAATACCTACG	GGATGACTGTGAGACAGCGA
TNFα	GCCTCTTCTCATTCCTGCTTG	CTGATGAGAGGGAGGCCATT
IL-1β	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT
IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA

Total Protein Extraction

Dissected hippocampus and frontal cortex were homogenized in TRIzol™ and total protein extracts were obtained from TRIzol-chloroform fractions, as described in (Simoes et al., 2013). Basically, TRIzol-chloroform fractions were transferred for 2 ml tubes and 100% ethanol was added to precipitate DNA. After mixing by inversion, the tubes were centrifuged at 2,000 *g* for 5 min at 4ºC. The supernatant, containing the proteins, was transferred to new 2 ml tubes and the DNA pellet discarded. Subsequently, isopropanol was added and the samples were incubated for 10 min at room temperature. Subsequently, samples were centrifuged at 12,000 *g* for 10 min and the supernatant discarded. Finally, the protein pellets were washed three times with 0.3 M guanidine-HCl in 95 % ethanol. In each wash, tubes were vigorously shaken until the pellets were dislodged from the tubes' walls, incubated at room temperature for 20 min, and then followed by centrifugation of 7,500 *g* for 5 min at 4ºC. After the final wash and centrifugation, 100% ethanol was added, and samples incubated at room temperature for 20 min, followed by centrifugation at 7,500 *g* for 5 min at 4ºC. The supernatant was discarded and the protein pellet resuspended in 8 M Urea (in Tris-HCl 1 M, pH 8.0) and 1% SDS solution (1:1), followed by 5 cycles of 15 s ultrasonication, with 2-3 min ice incubation between each cycle (sonication adjusted to 80% amplitude and 90% pulse – model UP100H, Hielscher Ultrasonics GmbH – 100

watts, ultrasonic frequency 30 Hz). Finally, the samples were centrifuged at 3,200 *g* for 10 min at 4ºC to sediment insoluble particles. The supernatants, containing the solubilized proteins, were recovered and stored at -80ºC.

Western Blot Analysis

Total protein extracts from hippocampus and frontal cortex, obtained from TRIzol-chloroform fractions (see above) were used. Protein concentrations were calculated using the Bio-Rad protein assay kit, according to the manufacturer's recommendations. Equal amounts of protein (60 µg) were electrophoretically resolved on denaturing 8 or 12% polyacrilamide gels. To evaluate Aβ and APP levels with the corresponding cleavage products (sAPP-β and CTF-β), 60 µg of total protein extracts were electrophoretically separated in 10–20% Tris–Tricine gels (Bio-Rad). The resolved proteins were transferred onto nitrocellulose membranes and blocking was performed with a 5% milk solution. Membranes were then incubated overnight with the following primary antibodies: rabbit polyclonal anti-p-APP (Thr668) antibody (3823; Cell Signalling); rabbit polyclonal anti-APP, C-terminal antibody (A8717; Sigma-Aldrich); mouse monoclonal anti-Aβ antibody (6E10; Covance), used to detect both total Aβ peptide and CFT-β; mouse monoclonal antibody anti-sAPP-β fragment with the Swedish mutation (sAPP-β; 6A1; Immuno-Biological Laboratories, Inc, Minneapolis, MN, USA); mouse monoclonal anti-GFAP antibody (GA5; Millipore Corporation, Temecula, CA, USA); rabbit polyclonal anti-p-Akt (Ser473) antibody (7985; Santa Cruz Biotechnology); rabbit polyclonal anti-Akt (8312; Santa Cruz Biotechnology); rabbit polyclonal anti-p-GSK3α/β (Ser21/9) (9331; Cell Signalling); mouse monoclonal anti-GSK3 α/β antibody (7219; Santa Cruz Biotechnology); rabbit polyclonal anti-p-tau (Ser396) (101815; Santa Cruz Biotechnology); mouse monoclonal anti-tau (tau5; 58860; Santa Cruz Biotechnology). In the next day, the membranes were incubated with goat secondary antibodies conjugated with horseradish peroxidase anti-mouse or anti-rabbit (BioRad Laboratories, Hercules, CA, USA) for 2 h at room temperature. After rinsing with TBS/0.2% Tween 20 three times (10 min each), the immunoreactive proteins were visualized with Immobilon™ Western (Millipore) or SuperSignal West Femto substrate (Thermo Scientific). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (32233; Santa Cruz Biotechnology) or β-actin (AC-15; Sigma-Aldrich) were

used as loading controls. Densitometric analyses were performed with the Image Lab software Version 5.1 Beta (Bio-Rad).

Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

Sandwich ELISA kits (Millipore) were used to determine total $A\beta_{1-40}$ and $A\beta_{1-42}$ concentrations from protein extracts of hippocampus and frontal cortex obtained from TRIzol-chloroform fractions. TRIzol contains guanidine isothiocyanate, a strong denaturing agent, which allows for the disruption of aggregated Aβ and subsequent recovery of total Aβ present in both soluble and insoluble fractions. The kits contain 96-wells (divided in 12 strips with 8 wells each) coated with a monoclonal antibody specific for the C-terminal end of either $A\beta_{1-40}$ or $A\beta_{1-42}$. In the first incubation step, 50 µl of appropriately diluted protein lysates were added to the wells, along with a biotin conjugated antibody that binds the N-terminal epitope of the immobilized Aβ. After orbital shaking for 5 min at 500 rpm/min, the samples were incubated overnight (16-20h) at 4ºC, without shanking. In the next day, solutions were decanted from the wells by tapping on paper, followed by washing of the wells 5 times with a washing solution. Afterwards, a peroxidase-streptavidin conjugate was added to each well for 30 min at room temperature on an orbital shaker (500 rpm), which allows the linking of peroxidase over a streptavidin-biotin bridge to the antibody-Aβ-antibody sandwich structure. After another washing step as described above, a peroxidase substrate is added, which is then enzymatically processed into a blue colored product, with the color intensity being proportional to the quantity of Aβ contained in sandwich structures. Next, the samples were placed on an orbital shaker (500 rpm) until the development of blue color was visually detectable (5-10 min), followed by the addition of an acidic solution that stops the enzymatic reaction and turns the blue color into yellow. Finally, absorbance was read at 450 nm in a microtiter plate reader (model 680, Bio-Rad), while absorbance at 590 nm was discounted as the baseline. Aβ concentrations were calculated from the standard curves, which were processed in parallel with the brain samples. Two quality controls were also included, whose Aβ concentration values, as calculated from the standard curve, ought to fall within an expected range for the remaining quantifications obtained with the assay to be considered accurate.

Statistical Analysis

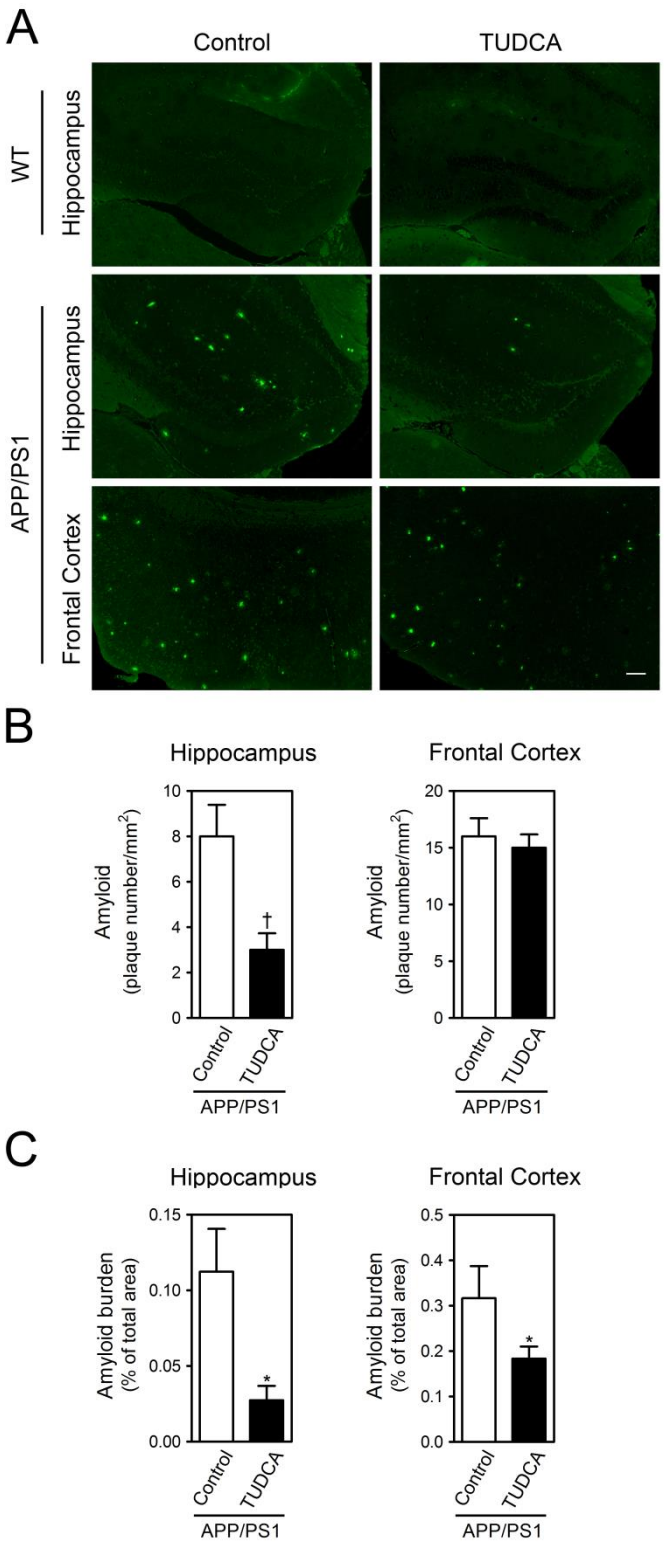
Data comparisons were conducted with one-way analysis of variance (ANOVA) followed by post hoc Bonferroni's test. Differences between two groups were analyzed by Student's two-tailed unpaired *t* test. Behavioral data obtained with the Morris water maze were analyzed with 2-way ANOVA for repeated measures (RM), followed by Tukey post hoc test. Analyses and graphical presentation were performed with the GraphPad Prism software Version 5 (GraphPad Software, Inc., San Diego, CA, USA). Results are presented as mean ± standard error of the mean (SEM).

III. RESULTS AND DISCUSSION

Short-term Treatment with TUDCA Attenuates Aβ Deposition in APP/PS1 Mice

APP/PS1 mice show increased Aβ production and parenchymal Aβ deposition beginning at 6 weeks of age, in the cortex, and at 2-3 months in the hippocampus (Radde et al., 2006). In a previous study, we demonstrated that TUDCA treatment started at initial phases of Aβ accumulation significantly prevented amyloid plaque development (Nunes et al., 2012). In the present study, we evaluated the effect of TUDCA on Aβ deposition when administrated after the onset of amyloid pathology in APP/PS1 mice, at 7 months of age. Thioflavin T staining (Fig. 1A) and 6E10 immunolabeling (Fig. 2A) revealed the presence of extensive amyloid deposits in the brains of APP/PS1 mice, which were not observed in wt mice. Importantly, amyloid plaque number was significantly decreased in the hippocampus of TUDCA-treated transgenic mice when compared to control transgenic mice ($p < 0.01$), whereas no difference was observed in the frontal cortex (Fig. 1B). Aβ immunohistochemistry further confirmed these results, showing ~ 50% reduction in Aβ plaque number in the hippocampus ($p < 0.05$) (Fig. 2B). Moreover, amyloid burden, as evaluated by Thioflavin T (Fig. 1C) and 6E10 staining (Fig. 2C) was decreased in the hippocampus and frontal cortex of TUDCA-treated APP/PS1 mice by ~ 65 and 40%, respectively ($p < 0.05$). The cortex is the first brain region affected by Aβ deposition in this APP/PS1 mouse model (Radde et al., 2006). It has been demonstrated that cortical formation of new amyloid plaques in this model occurs until 4-5 months of age and greatly decreases after this period, while both newly formed and existing plaques appear to grow at a similar rate (Hefendehl et al., 2011). Conversely, in the hippocampus, amyloid plaque formation only starts in the dentate gyrus at 2-3 months of age and in CA1 at 4-5 months of age (Radde et al., 2006), which might explain the differences observed in overall plaque burden and number between the frontal cortex and hippocampus with short-term TUDCA treatment (Fig. 1 and 2). Taken together, these results demonstrate that TUDCA treatment attenuates brain Aβ deposition after the onset of amyloid pathology, particularly in the hippocampus of APP/PS1 mice.

Fig. 1 TUDCA treatment reduces amyloid plaque pathology in the brains of APP/PS1. (A) Representative images of thioflavin staining in the hippocampus of control wt mice and hippocampus and frontal cortex of TUDCA-treated and untreated APP/PS1 mice. *Scale bar*, 100 µm. (B) Quantification of amyloid plaque number per square millimeter in hippocampus and frontal cortex of TUDCA-treated and control APP/PS1 mice. (C) Amyloid plaque burden presented as percentage of positive amyloid staining area relative to the total area of interest in hippocampus and frontal cortex of TUDCA-treated and untreated APP/PS1 mice. Values represent mean ± SEM of 6-7 mice per group. †p < 0.01 and *p < 0.05 from control APP/PS1 mice.



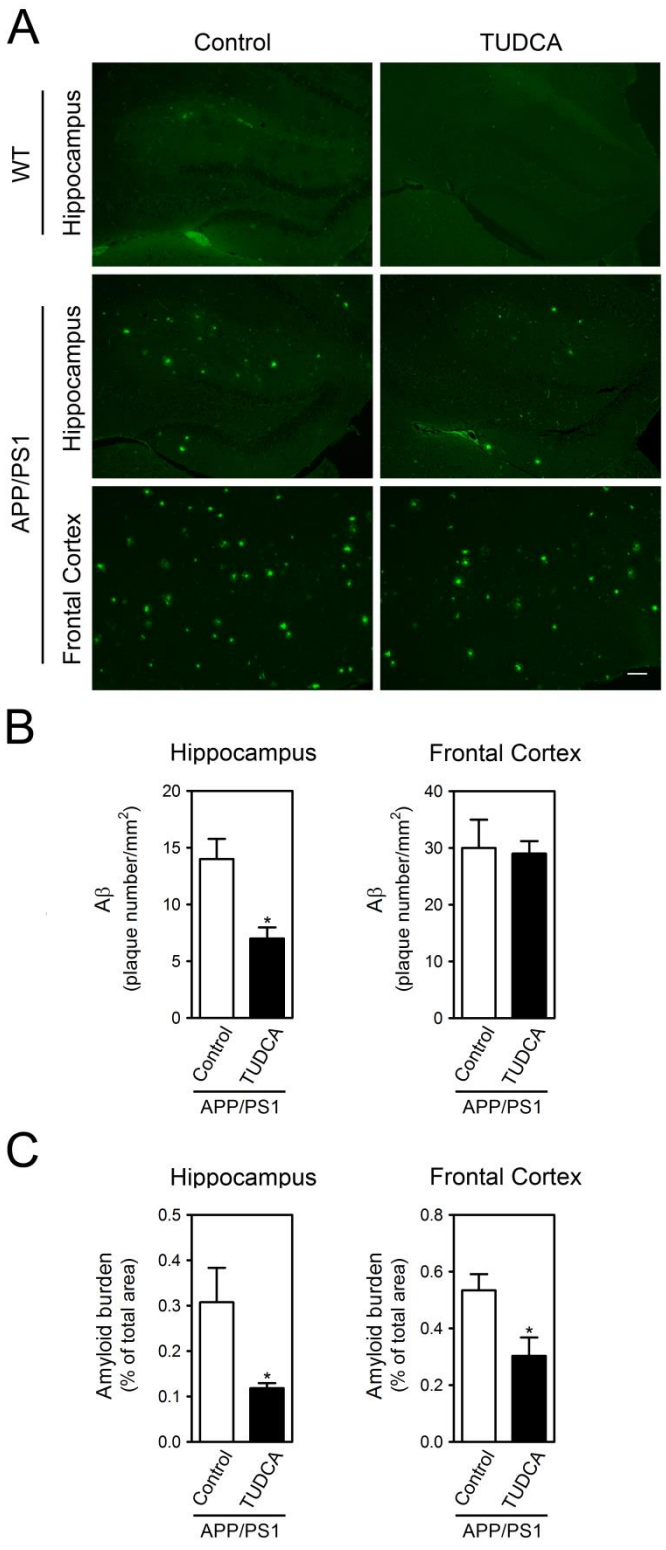


Fig. 2 TUDCA treatment reduces Aβ plaque pathology in the brains of APP/PS1 mice. (A) Representative images of Aβ immunostaining (6E10) in the hippocampus of control wt mice and hippocampus and frontal cortex of TUDCA-treated and untreated APP/PS1 mice. *Scale bar*, 100 μm. (B) Quantification of Aβ plaque number per square millimetre in hippocampus and frontal cortex of TUDCA-treated and control APP/PS1 mice. (C) Amyloid burden presented as percentage of positive Aβ immunoreactive area relative to the total area of interest in hippocampus and frontal cortex of TUDCA-treated and untreated APP/PS1 mice. Values represent mean ± SEM of 6-7 mice per group. **p* < 0.05 from control APP/PS1 mice.

TUDCA Reduces Amyloidogenic Processing of APP and Aβ Generation in APP/PS1 Mice

We have reported that preventive TUDCA treatment diminishes amyloidogenic processing of APP in APP/PS1 mice, suggesting that Aβ reduction by TUDCA is the main mechanism underlying attenuated amyloid pathology (Nunes et al., 2012). To

evaluate the effect of short-term treatment with TUDCA on the amyloidogenic processing of APP, we analyzed protein levels of APP β-secretase cleavage products, sAPP-β and CTF-β, by western blot (Fig. 3). Notably, sAPP-β fragment was significantly decreased both in the hippocampus and frontal cortex ($p < 0.05$) of TUDCA-treated APP/PS1 mice as compared to control APP/PS1 mice, while full-length APP levels remained unchanged. CTF-β levels were also reduced in the hippocampus ($p < 0.01$) and frontal cortex ($p < 0.05$) of TUDCA-treated transgenic mice when compared to controls. In agreement, total Aβ was also significantly decreased in the hippocampus ($p < 0.05$) and, to a lesser extent, in the frontal cortex ($p < 0.05$) of TUDCA-treated APP/PS1 mice, indicating that short-term treatment with TUDCA reduces the amyloidogenic processing of APP even at later stages of amyloid pathology.

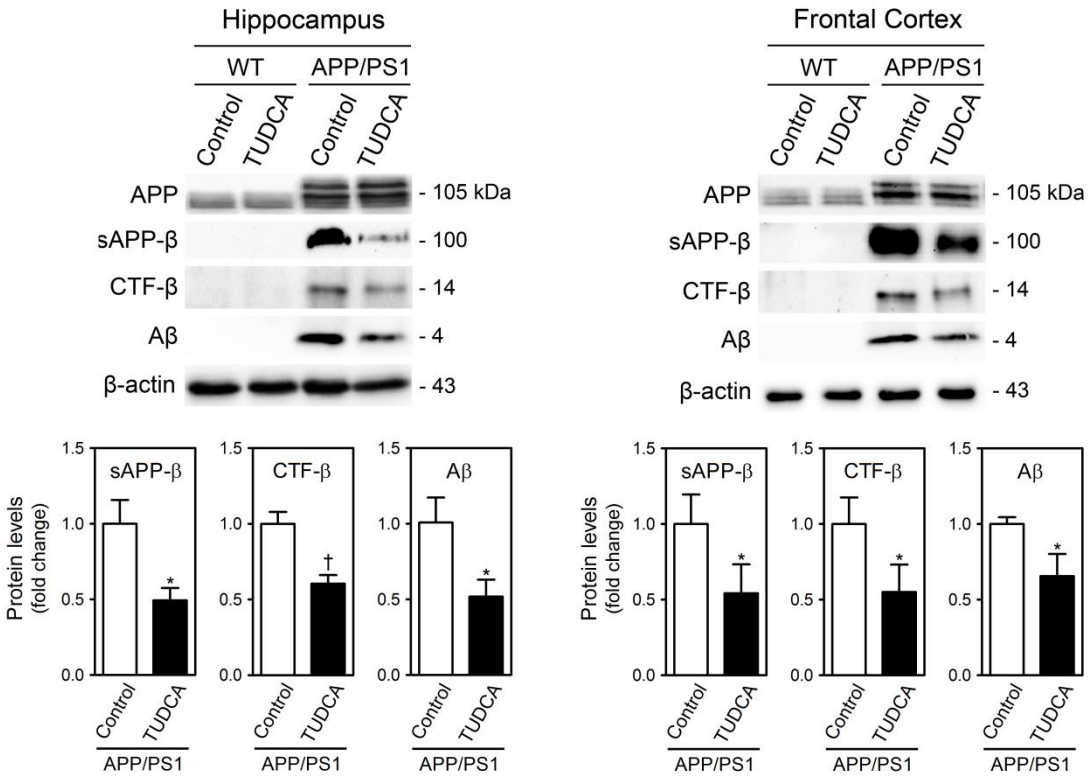


Fig. 3 TUDCA treatment decreases the amyloidogenic processing of APP in the brains of APP/PS1 mice, as evaluated by the production of sAPP-β, CTF-β and Aβ. Representative immunoblots of hippocampus and frontal cortex from control and TUDCA-treated wt and APP/PS1 mice with the respective densitometric analyses. β-actin was used as loading control. Values are expressed as mean ± SEM of 6-7 mice per group. †$p < 0.01$ and * $p< 0.05$ from control APP/PS1 mice.

We have also used sandwich ELISA to evaluate $A\beta_{42}$ and $A\beta_{40}$ levels in both brain regions of the four mice groups studied. As expected, wt mice presented undetectable levels of either $A\beta_{42}$ or $A\beta_{40}$ (data not shown). Further, we determined that both $A\beta_{42}$ and $A\beta_{40}$ levels were highly elevated in the brains of APP/PS1 mice, with $A\beta_{42}$ exceeding $A\beta_{40}$ by several-fold (Fig. 4). Importantly, TUDCA treatment resulted in a significant reduction of both Aβ species in the hippocampus and frontal cortex of APP/PS1 mice, when compared to untreated transgenic mice ($p < 0.05$). These findings indicate that TUDCA interferes with Aβ generation by reducing the amyloidogenic processing of APP, after the onset of the disease, thus representing a potential alternative for the existing therapeutic options.

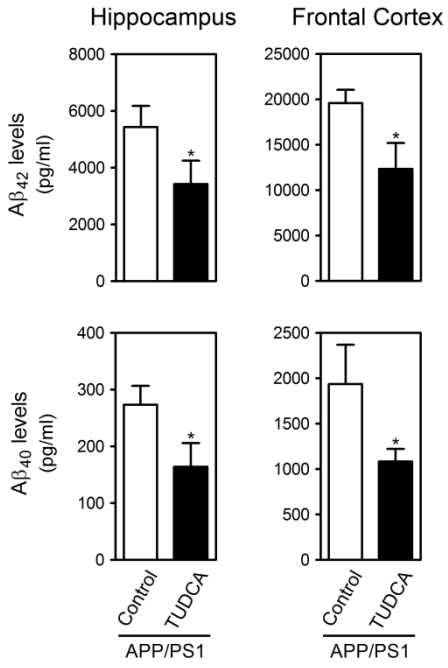


Fig. 4 TUDCA reduces $A\beta_{42}$ and $A\beta_{40}$ levels in the brains of APP/PS1 mice, as determined by ELISA. Quantification of $A\beta_{42}$ and $A\beta_{40}$ levels in the hippocampus and frontal cortex of control and TUDCA-treated APP/PS1 mice. Data are expressed as mean ± SEM of 6-7 mice per group. * $p < 0.05$ from control APP/PS1 mice.

TUDCA Supplementation Alters Akt/GSK3β Activities and Prevents tau Hyperphosphorylation in APP/PS1 Mouse Brains

The Akt/GSK3 pathway is known to be deregulated in AD (Ryder et al., 2004; Malm et al., 2007; Lee et al., 2009; Durairajan et al., 2012). The serine/threonine

kinase Akt is fully activated by phosphorylation at serine 473, after other downstream effects mediated by the phosphatidylinositol signalling pathway (Beaulieu et al., 2009). In turn, GSK3β is a substrate of Akt that is inhibited by phosphorylation of serine 9 (Beaulieu et al., 2009). In line with these findings, we determined the levels of phosphorylated Akt (p-Akt) and GSK3β (p-GSK3β) in brain lysates from TUDCA-treated and untreated APP/PS1 mice. Consistent with others (Durairajan et al., 2012), phosphorylation of Akt was significantly decreased in the frontal cortex of control APP/PS1 mice relative to wt mice ($p < 0.05$) (Fig. 5A). Noteworthy, the reduction in p-Akt levels was reverted in TUDCA-treated transgenic mice ($p < 0.05$), indicating increased activity of Akt with TUDCA treatment. Unexpectedly, our results showed no significant differences between Akt phosphorylation levels in the hippocampus of control APP/PS1 and control wt mice. In contrast, a trend to increase was observed in p-Akt levels of TUDCA-treated wt mice relative to control wt littermates, while a significant increase was detected between TUDCA-treated and control APP/PS1 mice ($p < 0.05$), indicating that TUDCA can specifically activate Akt in the hippocampus of wt mice.

Regarding p-GSK3β levels, they were significantly reduced in both the hippocampus ($p < 0.01$) and frontal cortex ($p < 0.005$) of control transgenic mice relative to wt controls (Fig. 5B). Importantly, TUDCA treatment reverted the reduction observed in p-GSK3β levels in APP/PS1 mice for both brain regions analyzed ($p < 0.005$). Our findings suggest that TUDCA may abrogate AD-associated GSK3β dysregulation, probably by specifically activating the upstream Akt signalling pathway.

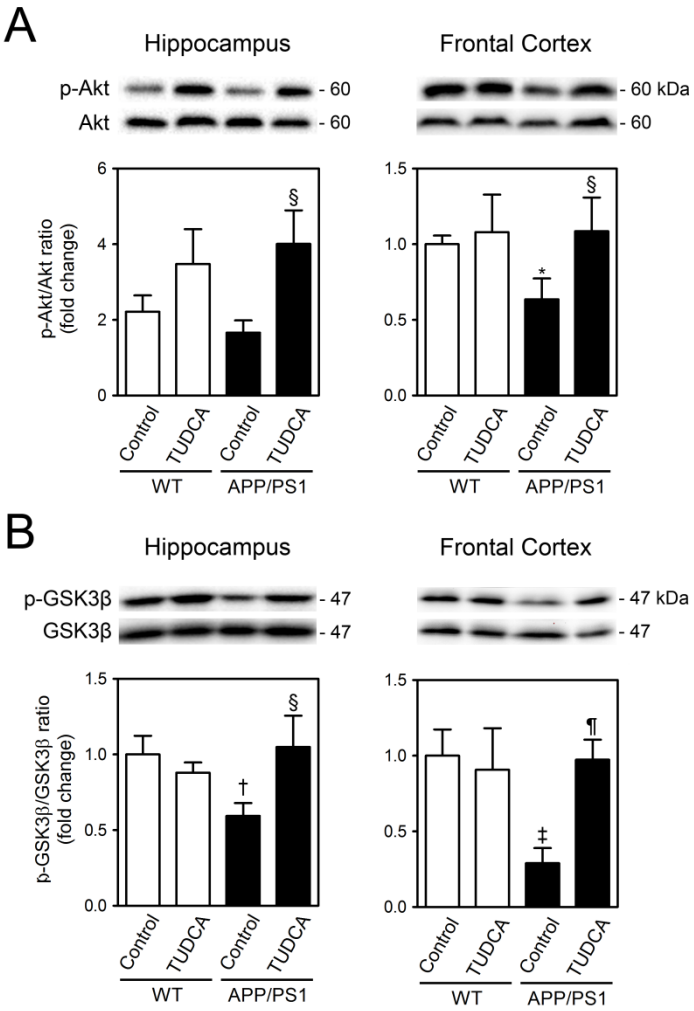


Fig. 5 TUDCA treatment increases Akt and GSK3β phosphorylation levels in the brains of APP/PS1 mice. (A) Representative immunoblots of p-Akt (Ser473) and total Akt in the hippocampus and frontal cortex of control and TUDCA-treated wt and APP/PS1 mice, and respective densitometric analyses of the p-Akt/Akt ratio. (B) Representative immunoblots of p-GSK3β (Ser9) and total GSK3β in the hippocampus and frontal cortex of control and TUDCA-treated wt and APP/PS1 mice with the respective densitometric analyses of the p-GSK3β/GSK3β ratio. Values are expressed as mean ± SEM of 6-7 mice per group. **p* < 0.05, †*p* < 0.01 and ‡ *p*< 0.005 from control wt mice and §*p* < 0.05 and ¶*p* < 0.01 from control APP/PS1 mice.

Interestingly, it has recently been reported that specific GSK3β inhibition resulted in decreased BACE1 mRNA expression and protein levels in an AD mouse model (Ly et al., 2013). Other authors have also described increased levels of BACE1 in AD patient brains (Yang et al., 2003). Taking this into account, we evaluated if TUDCA modulation of the amyloidogenic processing of APP was correlated with the

bile acid effect on GSK3β activity and, subsequently, BACE1 expression levels. However, this was not the case since no changes were detected in BACE1 levels after TUDCA treatment, at both mRNA and protein levels (Fig. 6).

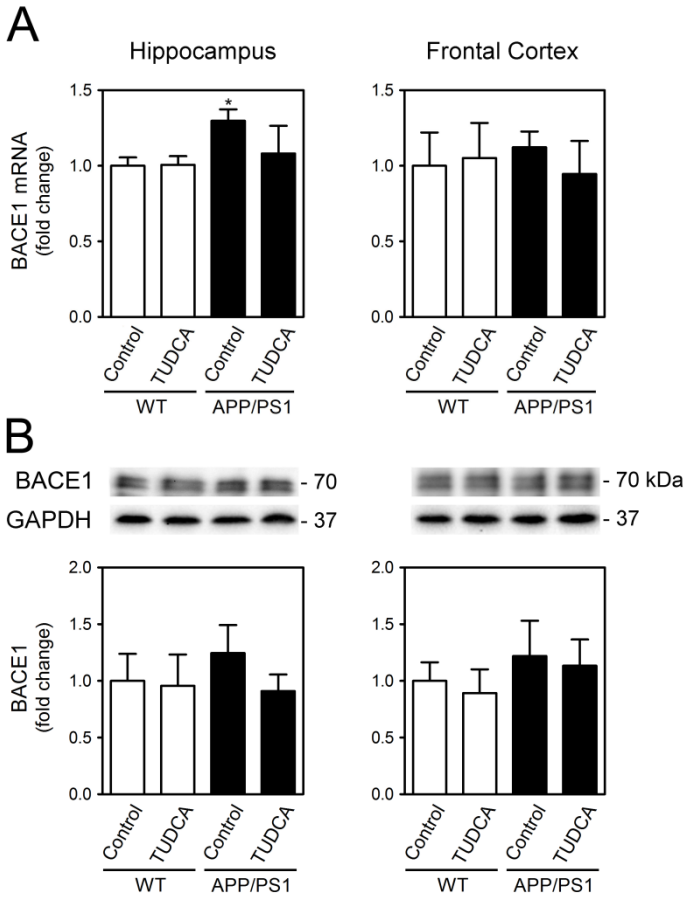


Fig. 6 TUDCA treatment does not affect BACE1 mRNA and protein levels in the brains of APP/PS1 mice. (A) BACE1 mRNA levels in the hippocampus and frontal cortex of control and TUDCA-treated wt and APP/PS1 mice, as determined by qRT-PCR. *p < 0.05 from control wt mice (n = 6-7 mice per group). (B) Representative immunoblots of BACE1 in hippocampus and frontal cortex from control and TUDCA-treated wt and APP/PS1 mice with the respective densitometric analyses. GAPDH was used as loading control. Values are expressed as mean ± SEM of 6-7 mice per group.

Alternatively, other studies *in vitro* and *in vivo* suggest that GSK3β is able to phosphorylate APP at threonine 668 (p-APP), which increases Aβ production (Aplin et al., 1996; Lee et al., 2003; Durairajan et al., 2012) Therefore, we hypothesized that the effect of TUDCA on GSK3β activity impacts on APP phosphorylation levels. Immunoblot analyses revealed that p-APP levels were increased in the frontal cortex of APP/PS1 mice comparing to wt mice ($p < 0.05$); however, they were unaffected by

TUDCA treatment (Fig. 7A). We further analyzed APP phosphorylation by immunohistochemistry and observed extensive accumulation of p-APP in dystrophic neurites closely associated with amyloid plaques, while total APP immunoreactivity was detected in both dystrophic neurites and neuronal perikaria (Fig. 7B). However, no changes were detected with TUDCA (Fig. 7B). These results are in agreement with previous studies in AD patient brains and in mouse models (Lee et al., 2003) (Shin et al., 2007). Curiously, p-APP immunoreactivity around amyloid plaques was more extensively detected in the frontal cortex than in the hippocampus of APP/PS1 mice (Fig. 7B).

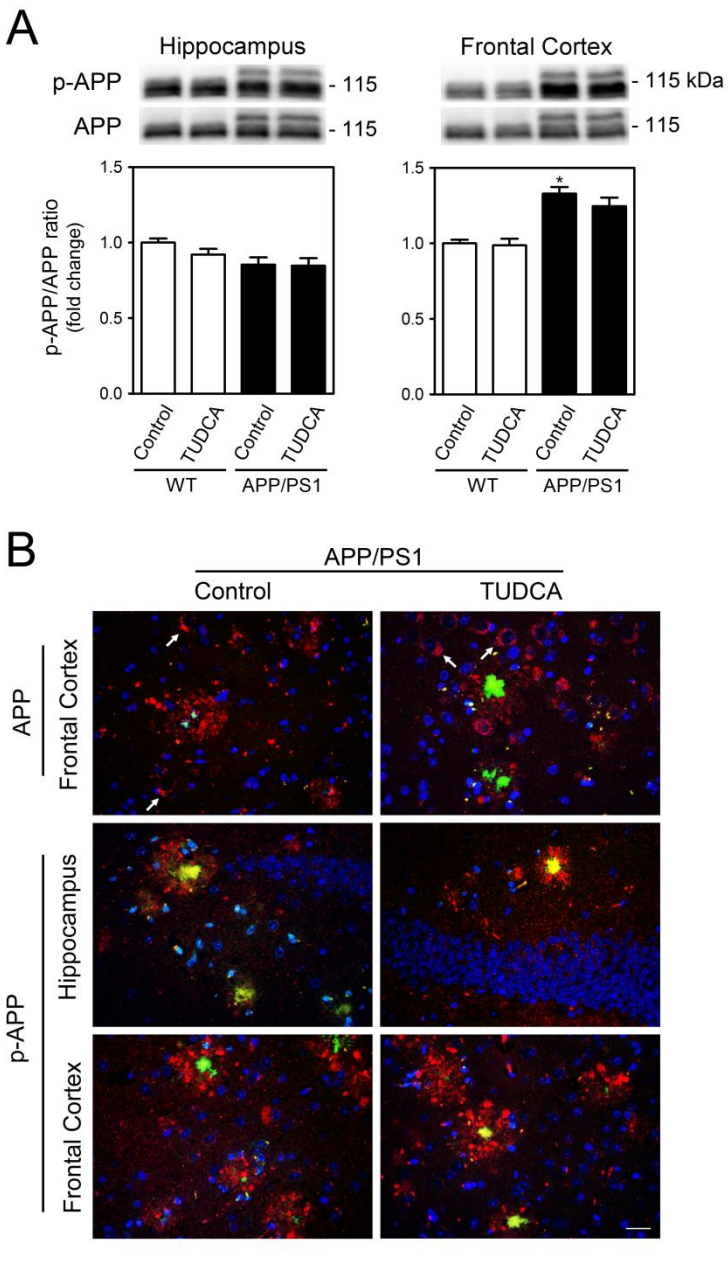


Fig. 7 TUDCA treatment does not alter APP phosphorylation in the brains of APP/PS1 mice. (A) Representative immunoblots of p-APP (Thr668) and total APP in hippocampus and frontal cortex lysates from control and TUDCA-treated wt and APP/PS1 mice with the respective densitometric analyses of the ratio p-APP/APP. Values are expressed as mean ± SEM of 6-7 mice per group. *$p < 0.05$ from control wt mice. (B) Fluorescent microscopy of amyloid plaques (thioflavin T-green) and APP or p-APP (red) in control and TUDCA-treated APP/PS1 mice. Cell nuclei were counterstained with Hoechst 33583 (blue). Unlike p-APP staining, which was mostly associated with dystrophic neurites located close to amyloid plaques, total APP was also extensively detected in neuronal perikarya (arrowheads). *Scale bar*, 25 µm.

In respect to tau, it is known that the APP/PS1 mouse model used in this study present hyperphosphorylated tau-positive neuritic structures located in the proximity of amyloid plaques at 8 months of age, with no significant NFT formation (Radde et al., 2006). Since GSK3β is one of the major kinases implicated in tau hyperphosphorylation (Li and Paudel, 2006; Leroy et al., 2010), we evaluated whether TUDCA-dependent inhibition of GSK3β was affecting p-tau levels. Tau phosphorylation was determined by immunoblotting and immunohistochemistry using an antibody against tau phosphorylated at Ser396 (corresponding to Ser936 of mouse origin), a residue already identified as being specifically phosphorylated by GSK3β in the absence of priming events (Li and Paudel, 2006; Leroy et al., 2010). As depicted in Fig. 8A, p-tau levels were increased ~2-fold in both hippocampus and frontal cortex ($p < 0.05$) of APP/PS1 mice relative to control wt mice. In contrast, TUDCA-treated transgenic animals presented a strong reduction in p-tau levels in both brain regions, showing a decrease of ~ 40% relative to control APP/PS1 mice ($p < 0.05$). Immunohistochemical analyses further revealed the presence of phosphorylated tau associated with dystrophic neurites surrounding amyloid plaques (Fig. 8B). Quantification of phosphorylated tau immunoreactivity further confirmed these results, supporting the view that the inhibitory effect of TUDCA on GSK3β activity positively impacts on tau hyperphosphorylation.

TUDCA Treatment Ameliorates Astrocytosis and Microgliosis in APP/PS1 Mice

Amyloid deposition leads to extensive microgliosis and astrocytosis surrounding the affected areas in AD patients and mouse models (Selkoe, 2001; Meraz-Rios et al., 2013). We have already described that TUDCA supplementation mitigates the activation of glial cells in APP/PS1 mice (Nunes et al., 2012). Interestingly, GSK3β is emerging as a key enzyme involved in the regulation of pathways and transcription factors involved in microglial and astrocytic activation (Yuskaitis and Jope, 2009; Beurel and Jope, 2010; Koistinaho et al., 2011). Due to the multiple lines of evidence showing a TUDCA-dependent effect on both GSK3β phosphorylation levels and glial activation, we assessed astrocytosis by GFAP immunostaining and immunoblot

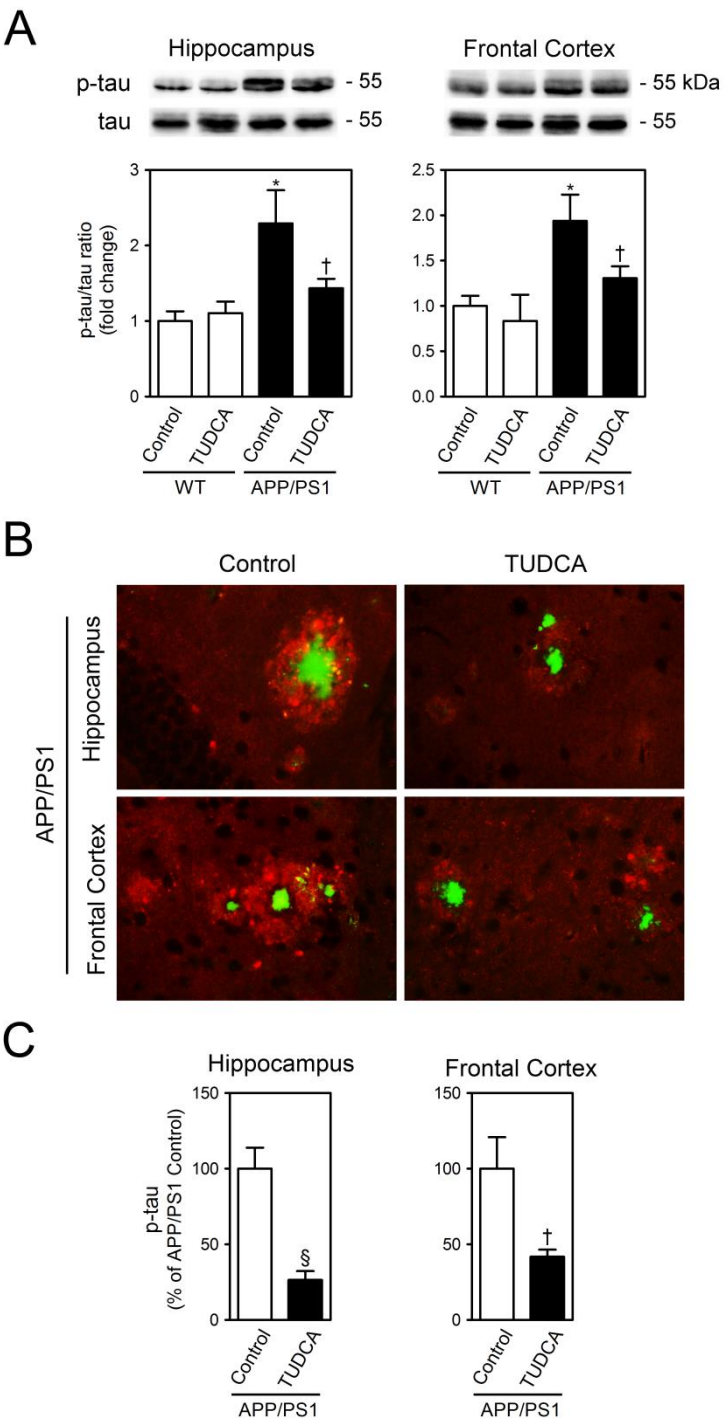


Fig. 8 TUDCA treatment reduces tau phosphorylation in the brains of APP/PS1 mice. (A) Representative immunoblots of p-tau (Ser396) and total tau in the hippocampus and frontal cortex of control and TUDCA-treated wt and APP/PS1 mice with the respective densitometric analyses of the p-tau/tau ratio. (B) Double staining of p-tau (red) and amyloid plaques (thioflavin T - green) in the hippocampus and frontal cortex of control and TUDCA-treated APP/PS1 mice. *Scale bar*, 25 µm. (C) Quantification of p-tau mean gray values in the hippocampus and frontal cortex of control and TUDCA-treated APP/PS1 mice, presented as percentage of control APP/PS1 mice. Values are expressed as mean ± SEM of 6-7 mice per group. *p < 0.05 from control wt mice and †p < 0.05 and §p < 0.01 from control APP/PS1 mice.

analyses, and microgliosis by Iba-I immunostaining. As expected, GFAP immunofluorescence showed a prominent increase of reactive astrocytes in the hippocampus ($p < 0.01$) and frontal cortex ($p < 0.005$) of control APP/PS1 mice compared to control wt mice (Fig. 9A and B). Noteworthy, GFAP-reactive astrocytes were significantly reduced in both brain regions of TUDCA-treated APP/PS1 mice compared to APP/PS1 controls ($p < 0.05$). Additionally, GFAP protein levels were also

evaluated by immunoblot, further confirming the dramatic increase in GFAP levels in the hippocampus ($p < 0.005$) and frontal cortex ($p < 0.001$) of control transgenic mice, when compared to control wt mice (Fig. 9C).

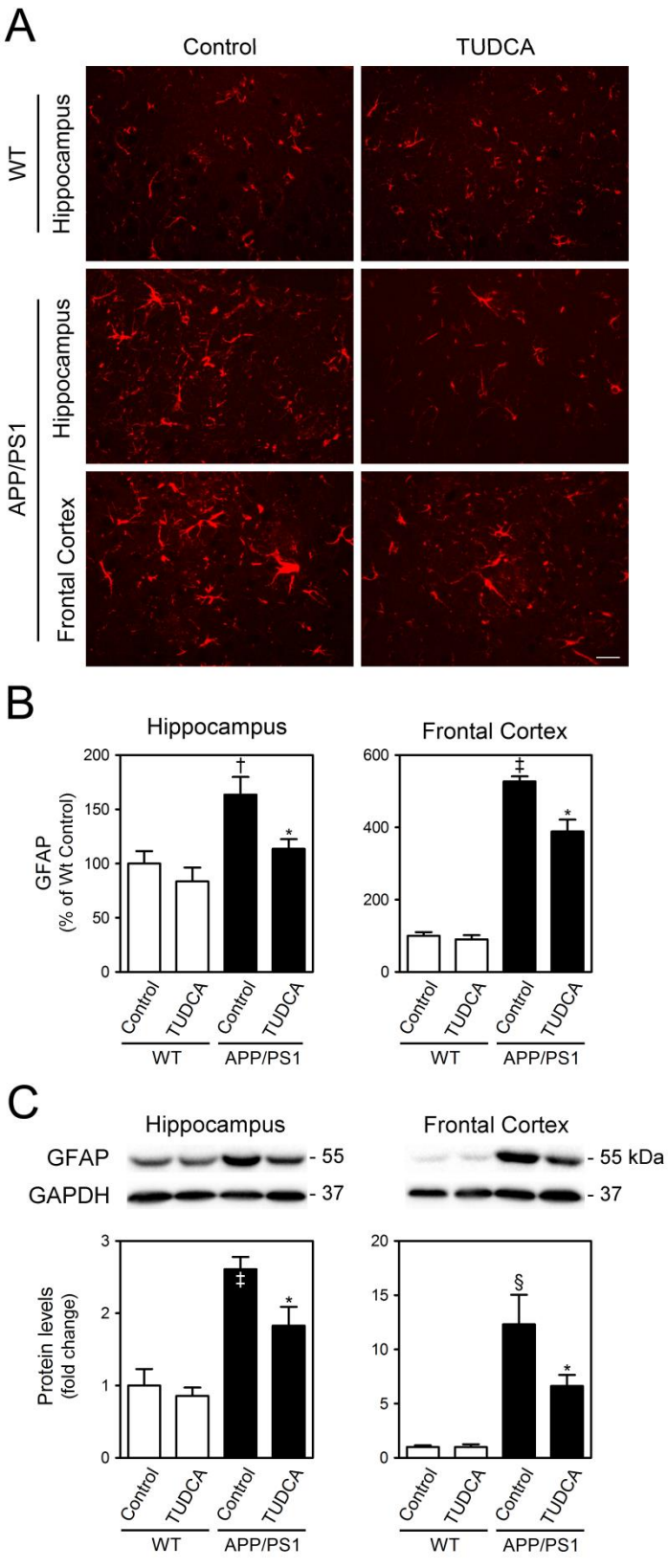


Fig. 9 TUDCA treatment attenuates astrogliosis in the brains of APP/PS1 mice. (A) Representative images of GFAP immunostaining in the hippocampus of control wt mice and hippocampus and frontal cortex of control and TUDCA-treated APP/PS1 mice. *Scale bar*, 25 µm. (B) Quantification of GFAP mean gray values in the hippocampus and frontal cortex of control and TUDCA-treated wt and APP/PS1 mice, presented as percentage of control wt mice. (C) Representative immunoblots of GFAP in hippocampus and frontal cortex lysates from control and TUDCA-treated wt and APP/PS1 mice with the respective densitometric analyses. GAPDH was used as loading control. Values are expressed as mean ± SEM of 6-7 mice per group. †$p < 0.01$, ‡$p < 0.005$ and §$p < 0.001$ from control wt mice and *$p < 0.05$ from control APP/PS1 mice.

Moreover, our results revealed a ~ 30% decrease in GFAP levels in the hippocampus and 45% in the frontal cortex of TUDCA-treated APP/PS1 mice relative to APP/PS1 controls ($p < 0.05$), further corroborating the results obtained for GFAP immunostaining (Fig. 9C).

Microgliosis was significantly elevated in the hippocampus and frontal cortex of control transgenic mice relative to wt mice ($p < 0.005$) (Fig. 10A and B). Importantly, TUDCA treatment of APP/PS1 animals decreased Iba-I immunofluorescence in both hippocampus and frontal cortex ($p < 0.01$). Since reactive microglia are usually tightly clustered around amyloid deposits, we performed double immunohistochemical staining with Iba-I and Thioflavin, to evaluate the effect of TUDCA in amyloid-dependent microgliosis. In accordance with previous reports, clusters of hypertrophic microglia were detected in close proximity to Aβ plaques in the brains of control APP/PS1 mice, while less reactive microglia were visualized surrounding amyloid plaques in the parenchyma of TUDCA-treated transgenic mice (Fig. 10C).

To further characterize the effect of TUDCA on the pro-inflammatory response, we analyzed by qRT-PCR the expression levels of TNF-α, IL-1β and IL-6, pro-inflammatory cytokines extensively described as being augmented in AD (Meraz-Rios et al., 2013). As anticipated, the mRNA levels of all three cytokines were significantly elevated in both hippocampus and frontal cortex of control APP/PS1 mice when compared to control wt mice (Fig. 11), with TNF-α mRNA in the frontal cortex presenting the highest increase (~ 11-fold, $p < 0.0001$). TNF-α mRNA levels were significantly decreased by TUDCA treatment both in the hippocampus ($p <0.05$) and in the frontal cortex ($p < 0.005$) of APP/PS1 mice relative to control APP/PS1 mice. IL-1β and IL-6 mRNA levels showed no significant differences or a trend to decrease between these two groups. These results further indicate that TUDCA reduces neuroinflammation in APP/PS1 mice.

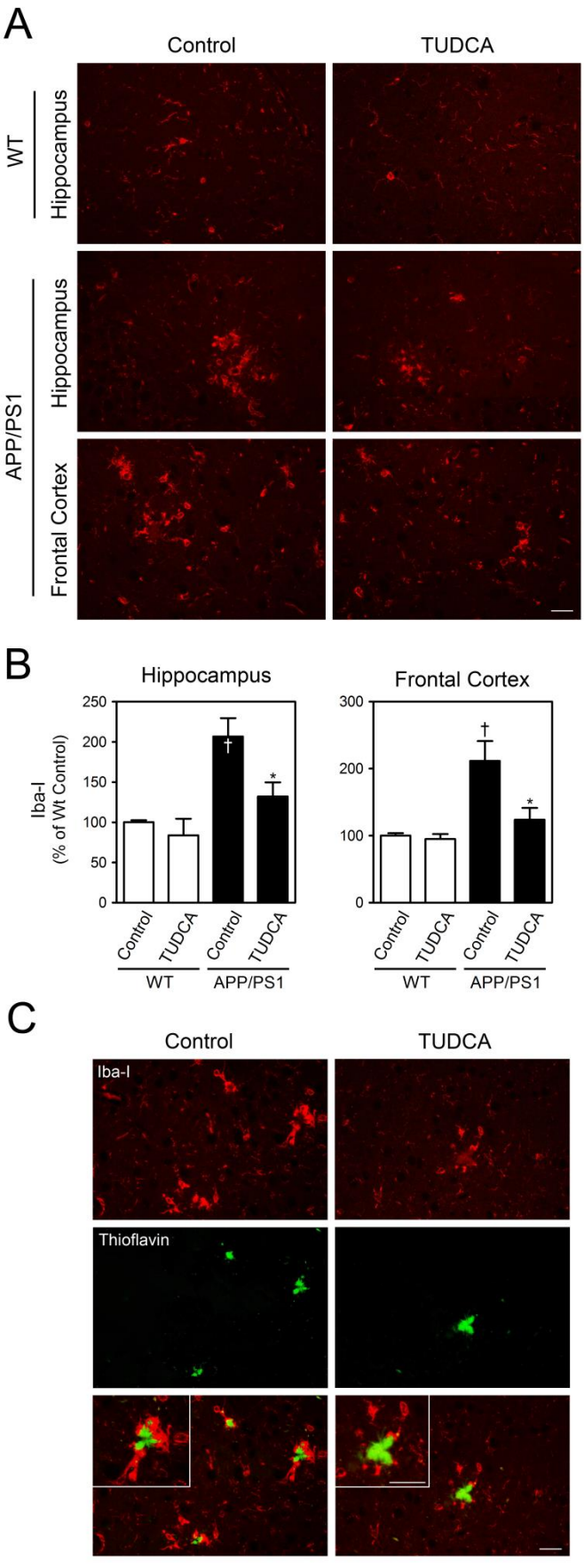


Fig. 10 TUDCA treatment reduces microgliosis in the brains of APP/PS1 mice. (A) Representative images of Iba-I immunostaining in the hippocampus of control wt mice and hippocampus and frontal cortex of control and TUDCA-treated APP/PS1 mice. *Scale bar*, 25 µm. (B) Quantification of Iba-I MGV in the hippocampus and frontal cortex of control and TUDCA-treated wt and APP/PS1 mice, presented as percentage of control wt mice. Values are expressed as mean ± SEM of 6-7 mice per group. †$p < 0.005$ from control wt mice and *$p < 0.01$ from control APP/PS1 mice. (C) Double staining of Iba-I and amyloid plaques (thioflavin T staining) in the hippocampus of control and TUDCA-treated APP/PS1 mice. *Scale bar*, 25 µm

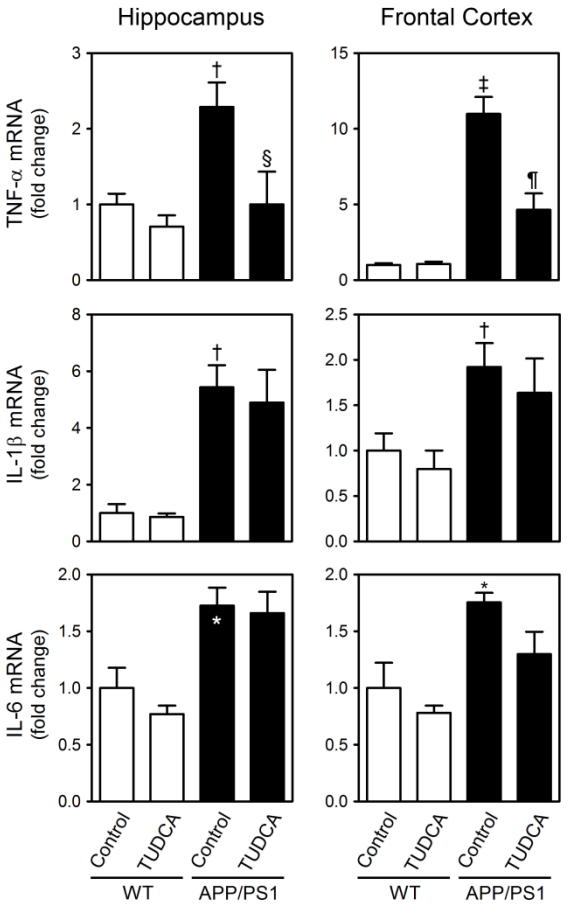


Fig. 11 TUDCA treatment decreases TNF-α mRNA expression in the hippocampus and frontal cortex of APP/PS1 mice. Quantification of TNF-α, IL-1β and IL-6 mRNA levels in the hippocampus and frontal cortex of control and TUDCA-treated wt and APP/PS1 mice. Data are expressed as mean ± SEM of 6-7 mice per group. *$p < 0.01$, †$p < 0.005$, ‡$p < 0.001$ from control wt mice and §$p < 0.05$ and ¶$p < 0.005$ from control APP/PS1 mice.

TUDCA treatment reduces synaptic loss in APP/PS1 mice

Aβ species and extensive gliosis have been widely implicated in the neurodegenerative processes observed during AD progression. However, several transgenic mouse models of AD only manifest global neuronal loss at very advanced ages (~ 17 months of age) (Wirths and Bayer, 2010). Further, the APP/PS1 mouse model used in this study only manifest a modest but significant neuron loss (11%) at 17 months of age in high neuronal density subregions, such as the granule cell layer of the dentate gyrus (Rupp et al., 2011). Furthermore, most neurons that present dystrophic axons associated with amyloid plaques are still viable (Adalbert et al., 2009). These features thus render our mouse model inappropriate to study the anti-apoptotic properties of any given substance in AD (Radde et al., 2006; Rupp et al., 2011). On the other hand, several animal models present loss of presynaptic terminals, as determined by SYN immunostaining and protein levels (Radde et al., 2006; Ding et al., 2008; Nunes et al., 2012). Interestingly, soluble Aβ oligomers are widely accepted as the main neurotoxic species, directly contributing to loss of synaptic integrity and

subsequently to memory impairment and cognitive deficits (Selkoe, 2002). Synapse loss starts as early as at 5 months of age in the hippocampal dentate gyrus (Ding et al., 2008), indicating that APP/PS1 mice models mimic the progression of synaptic dysfunction that is observed early in patients with very mild to mild AD (Masliah et al., 2001; Selkoe, 2002). The hippocampal dentate gyrus is highly enriched in synaptic connections between the mossy fibers of the dentate gyrus and the dendrites of the pyramidal neurons; further, the synaptic connections of this brain region are commonly affected in APP/PS1 mice in an age-related manner, even in regions devoid of amyloid plaques, which is thought to relate to the development of memory deficits usually observed in these models (Rutten et al., 2005; Ding et al., 2008). Consequently, we proceeded to evaluate synaptic integrity in the dentate gyrus by SYN immunostaining. As anticipated, semiquantitative analysis of SYN immunostaining revealed an accentuated decrease in SYN immunoreactivity in the polymorphic layer of the dentate gyrus of control APP/PS1 mice relative to control wt mice ($p < 0.005$), while TUDCA treatment partially reverted this phenotype in APP/PS1 mice ($p < 0.05$) (Fig. 12). These data indicate that TUDCA administration even after the onset of AD pathology can still partially protect from loss of synaptic function.

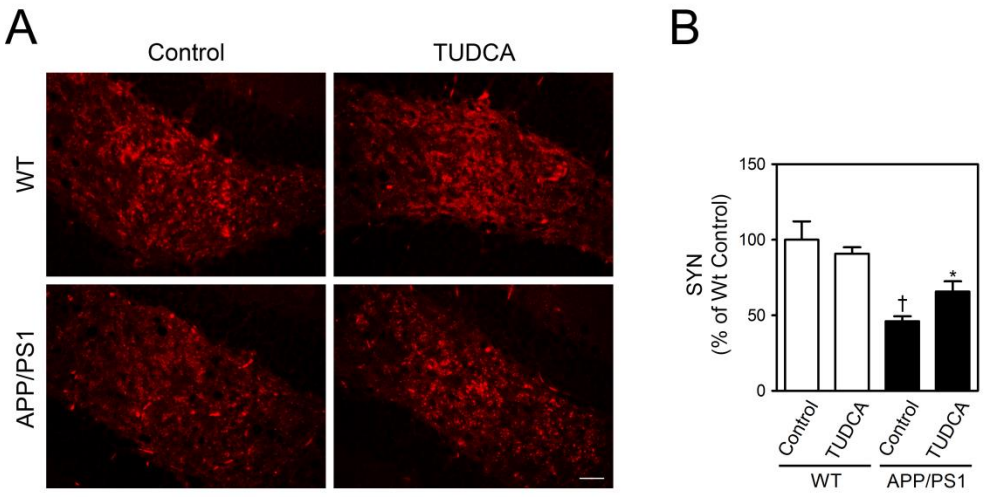


Fig. 12 TUDCA treatment partially rescues loss of presynaptic terminals. (A) Representative images of SYN immunostaining in the hippocampus of control and TUDCA-treated wt and APP/PS1 mice. *Scale bar*, 25 µm. (B) Quantification of SYN mean gray values in the hippocampus of control and TUDCA-treated wt and APP/PS1 mice, presented as percentage of control wt mice. Values are mean ± SEM of 6-7 mice per group. †$p < 0.005$ from control wt mice and *$p < 0.05$ from control APP/PS1 mice.

APP/PS1 double-transgenic mice undergo memory deterioration due to the age-associated progression of Aβ pathology (Radde et al., 2006). To evaluate whether TUDCA administration ameliorates memory deficits in aged APP/PS1 mice, all animals were trained in the Morris water maze. During training, we measured no significant differences between groups. All groups learned to locate the hidden platform position over consecutive training days (Two-way RM ANOVA: *F9*, 351 = 82.96, $p < 0.001$) and showed similar learning curves (Fig. 13A). During the second probe trial, we removed the platform and measured the time mice spent in each of the different quadrants. We found a significant quadrant (Two-way RM ANOVA: *F3*, 117 =24.59, $p < 0.001$) and quadrant by group effect (*F9*, 117 = 2.144, $p < 0.05$). Post hoc test shows that both wt groups spent a significant amount of time in the target quadrant ($p < 0.001$), whereas such a target quadrant preference is lacking in control APP/PS1 mice (Fig. 13B). Nevertheless, a trend to improved memory was detected, which probably correlates with TUDCA-dependent attenuation of Aβ pathology.

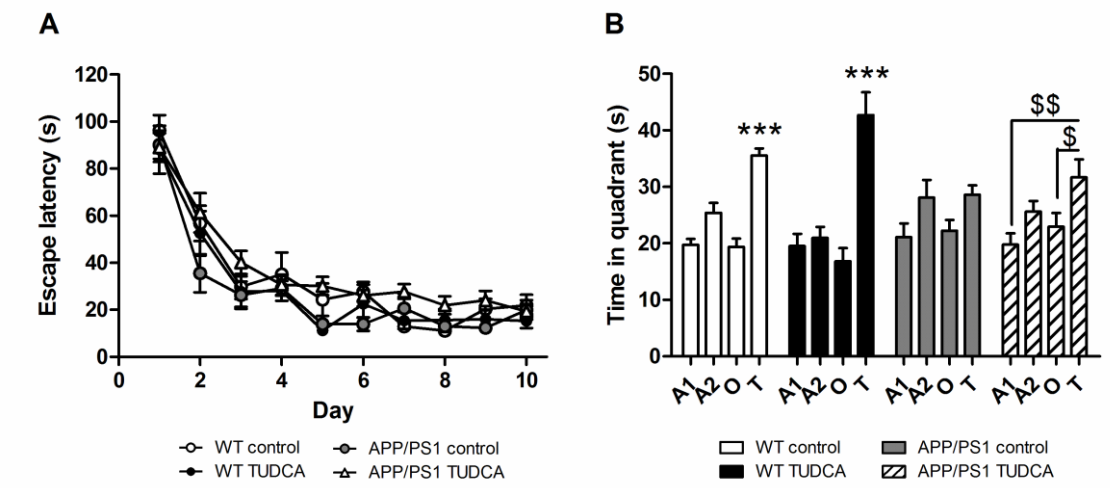


Fig. 13 Cognitive performance in the Morris water maze in vehicle and TUDCA-treated wt and APP/PS1 mice. (A) During training, no significant group differences were measured in time to find the hidden platform. All groups showed similar learning curves. (B) During the second probe trial, we found a significant target quadrant preference in both control and TUDCA-treated wt mice. Such preference was not found in control APP/PS1 mice. TUDCA-treated APP/PS1 mice showed a trend to target quadrant preference, albeit not significant (n = 10 control wt mice; n = 8 TUDCA-treated wt mice; n = 11 control APP/PS1 mice; and n = 14 TUDCA-treated APP/PS1 mice). Data are expressed as means ± SEM. *$p < 0.001$ between target quadrant and all other quadrants; †$p < 0.05$ between target quadrant and A1 quadrant and ‡ $p < 0.01$ between target quadrant and opposite quadrant. A1 and A2: adjacent quadrants, O: opposite quadrant, T: target quadrant.

IV. DISCUSSION

AD is the most prevalent neurodegenerative disorder in ageing populations worldwide. Available treatments are mostly symptomatic and unable to arrest or revert the progression of the disease (Reitz and Mayeux, 2014). In this regard, we have recently described a remarkable rescue of different memory types, as well as improved synaptic efficiency in APP/PS1 transgenic mice long-term treated with neuroprotective TUDCA (Nunes et al., 2012; Lo et al., 2013; Ramalho et al., 2013). Now, we extended our studies and further demonstrated the therapeutic efficacy of TUDCA when administrated at 7 months of age, after the onset of amyloid pathology. The relevance of using transgenic mouse models to study AD has raised several concerns, since most of the clinical trials using AD therapeutic agents tested in mice result in negative therapeutic outcomes (Kokjohn and Roher, 2009). Nevertheless, animal models, including APP/PS1 mice, remain instrumental for dissecting the molecular pathways involved in AD and evaluating new therapeutic avenues *in vivo*.

Notably, our results suggest that, although TUDCA treatment does not completely revert amyloid pathology after its establishment, this bile acid can still attenuate the progression of Aβ deposition, as confirmed by the diminished production of APP cleavage fragments and decrease of total Aβ levels in the hippocampus and frontal cortex of APP/PS1 mice.

We showed that Akt activity was reduced in the frontal cortex of APP/PS1 mice relative to control littermates, while no significant changes were observed in the hippocampus. However, GSK3β was hyperactivated in both brain regions, which indicates that hippocampal GSK3β deregulation is probably independent of the upstream Akt pathway. Although unexpected, the absence of differences in Akt phosphorylation levels in the hippocampus, with a concomitant decrease in GSK3β phosphorylation between wt and APP/PS1 mice has also been demonstrated by others in APP/PS1 mice at 16 months of age (Malm et al., 2007). Controversially, other studies have reported a reduction in both Akt and GSK3β phosphorylation levels in the hippocampus of 6 and 13 months-old APP/PS1 mice (Hu et al., 2013; Jia et al., 2013). Interestingly, AD patients appear to present reduced Akt phosphorylation in hippocampal neurons only at end stages of disease, when altered Akt activity is already established in the cortex (Griffin et al., 2005; Lee et al., 2009). This observation has been extended in other reports that detected reduced phosphorylation

of Akt in AD brain, along with GSK3 hyperactivity (Lee et al., 2009; Liu et al., 2011). This feature is widely mimicked *in vivo* by AD transgenic mouse models, and in *in vitro* by cell models harbouring familial AD mutations (Ryder et al., 2004; Durairajan et al., 2012; Jia et al., 2013; Jimenez et al., 2011;). Taken together, it is possible that hippocampal Akt deregulation correlates with affected neuronal integrity in this brain region, which can be detected in transgenic mice only several months after the development of other AD-related features.

We have previously shown that TUDCA can activate the PI3K/Akt signalling pathway, resulting in neuronal protection against Aβ toxicity (Sola et al., 2003). It is therefore plausible that the increased Akt phosphorylation levels detected in TUDCA-treated APP/PS1 and wt mice depends on the activation of upstream PI3K. However, the role of PI3K/Akt signalling in AD remains controversial. Several reports implicate insulin resistance as a probable mechanism towards impaired PI3K/Akt signalling and subsequent alterations in neuronal function, metabolism and survival (Liu et al, 2011; de la Monte, 2012; Lee et al, 2009). In fact, AD brain analyses revealed impaired insulin downstream signalling involving abnormal IRS-1 serine phosphorylation patterns that were correlated with reduced PI3K and/or Akt activity (Liu et al.,2011; Lee et al., 2009; Bomfim et al., 2012). Curiously, AD patients with type 2 diabetes presented a more severe reduction in the activity of insulin/PI3K/Akt than AD or diabetes patients, suggesting that deficient brain insulin signalling may precede AD and contribute to the progression of the disease (Liu et al., 2011). This observation further implicates diabetes as a risk factor for AD, and downstream PI3K/Akt inactivation might be involved in this process.

Interestingly, insulin-deficient diabetic mice also develop cognitive deficits and impaired memory, which is strongly correlated with decreased IR, Akt and GSK3 phosphorylation levels in the brain (Jolivalt et al., 2008). Moreover, mounting evidence suggest that soluble Aβo can bind to IR and block its activation, thus reducing insulin downstream signalling (Zhao et al., 2008; De Felice et al., 2009; Moloney et al., 2010). Aβ can also impair insulin signalling by activating TNF-α/JNK, which leads to abnormal IRS-1 phosphorylation and activity (Bomfim et al., 2008; Jia et al., 2013). Importantly, this was reported in an APP/PS1 mouse model that develops brain insulin resistance due to abnormal IRS-1 phosphorylation mediated by TNF-α/JNK. Moreover, treatment

with a natural polyphenol significantly reduced JNK activity and TNF-α levels, reverting PI3K/Akt/GSK3β signalling impairment and ameliorating cognitive deficits (Jia et al., 2013). These observations place Aβ pathology above insulin/PI3K/Akt deregulation. Since we have also detected reduced levels of TNF-α in the brains of TUDCA-treated APP/PS1 mice, it is tempting to speculate that this could be correlated with decreased JNK activation and, subsequently, with higher activity of the IRS-1/PI3K/Akt pathway. TUDCA can also downregulate Aβ-driven JNK activity, which further strengthen this hypothesis (Viana et al, 2010).

Conversely, others suggested that abnormal upregulation of the PI3K/Akt pathway in AD brain can lead to insulin resistance by feedback mechanisms, as well as other AD pathologies (Talbot et al, 2012; O'Neill, 2013). It has been suggested that one of the major mechanisms by which PI3K/Akt mediates AD progression, depends on mTOR activation and subsequent decrease in autophagy and upregulation of tau and APP expression (O'Neill, 2013). In this regard, further analyses should be performed to dissect the contribution of PI3K/Akt in the present study. Evaluation of IR levels, IRS1 and 2 patterns of phosphorylation, activation of JNK by phosphorylation, as well as other downstream events, such as Akt-mediated phosphorylation of mTOR and autophagy, would contribute to further characterize the insulin/PI3K/Akt pathway in APP/PS1 mice and its modulation by TUDCA (Talbot et al, 2012; O'Neill, 2013).

Importantly, the role of TUDCA treatment in the modulation of Akt/GSK3β signalling was highlighted by the reversion of altered Akt/GSK3β levels in the cortex, and GSK3β in the hippocampus, to control levels, suggesting a therapeutical effect. Moreover, inhibition of GSK3β activity correlated with a reduction in tau hyperphosphorylation levels at Ser396, suggesting that inactivation of GSK3β by TUDCA positively modulates AD-associated tau pathology.

Although GSK3β has been also widely accepted as a major player in several pathological mechanisms associated with AD, the mechanism by which this kinase regulates cerebral amyloidosis is still somewhat controversial. Some authors have reported that GSK3β inhibition downregulates *BACE1* gene expression in APP23/PS45 double transgenic mice (Ly et al., 2013), while others did not detected significant alterations in BACE1 protein levels (Ding et al., 2008; Durairajan et al., 2012). This discrepancy might be attributable to differences between the animal models used or

the ages at which the animals were engaged in the respective studies. Surprisingly, no significant changes were detected in BACE1 mRNA and protein levels between APP/PS1 mice and wt littermates, treated or untreated with TUDCA

GSK3β also appears to be involved in the phosphorylation of APP at the cytoplasmic residue Thr668, which is thought to elevate Aβ generation (Aplin et al., 1996; Lee et al., 2003; Chang et al., 2006; Durairajan et al., 2012). In this respect, we evaluated whether the effect of TUDCA on APP processing was dependent on the regulation of GSK3β-dependent APP phosphorylation. Immunoblot analysis revealed increased p-APP levels solely in the cortex of APP/PS1 mice, which was not altered by TUDCA administration. Still, there are other kinases deregulated in AD pathology, such asCDK5 and JNK, that are postulated to phosphorylate APP, which might explain the unaltered p-APP levels despite GSK3β inhibition (Durairajan et al., 2012). Several studies suggest that APP phosphorylation targets the protein for fast axonal transport to the nerve terminals. Increased co-localization of APP with β- and γ-secretases within axonal or presynaptic vesicles culminates in enhanced Aβ generation (Lee et al., 2003; Lee et al., 2005; Durairajan et al., 2012). In fact, p-APP immunoreactivity was mostly associated with dystrophic neurites located around amyloid plaques, while total APP immunoreactivity was observed in both dystrophic neurites and neuronal perikarya in the brains of APP/PS1 mice, indicating that p-APP is preferably located in axonal regions. Interestingly, p-APP immunoreactivity was more pronounced around amyloid plaques located in the frontal cortex than in those located in the hippocampus, indicating that the increase in p-APP levels as determined by immunoblot analysis in the frontal cortex probably derives from extensive p-APP accumulation around amyloid plaques. Increased p-APP levels in the cortex of APP/PS1 mice relative to the hippocampus are probably associated with the earlier onset of amyloid deposition observed in the cortex, although a region specific effect cannot be excluded. These findings indicate that TUDCA treatment does not influence APP processing by modulating BACE1 protein levels or APP phosphorylation levels.

Other aspect of GSK3 inhibition is the associated decrease of glial activation and overall neuroinflammatory markers (Yuskaitis and Jope, 2009; Beurel and Jope, 2010; Koistinaho et al., 2011). In this context, GSK3β appears to upregulate the expression of several pro-inflammatory mediators, such as TNF-α (Yuskaitis and Jope,

2009; Wang et al., 2010), while decreasing anti-inflammatory molecules (Koistinaho et al., 2011). Moreover, GSK3β inhibition reduces microglial activation and migration (Yuskaitis and Jope, 2009), and increases inflammatory tolerance in astrocytes upon repeated inflammatory stimuli, suggesting a paramount role for this kinase during chronic neuroinflammation (Beurel and Jope, 2010). As expected, a marked neuroinflammatory phenotype was evident in the brains of APP/PS1, with a significant increase in gliosis and upregulation of pro-inflammatory TNF-α, IL-1β and IL-6. Importantly, TUDCA administration after the onset of amyloid pathology significantly ameliorated astrocytosis and microgliosis in both hippocampus and frontal cortex of APP/PS1 mice, and specifically inhibited TNF-α mRNA expression. These results are in agreement with others showing decreased levels of TNF-α mRNA in activated astrocytes treated with GUDCA but no alterations in IL-1β expression (Fernandes et al., 2007). More importantly, anti-TNF-α therapeutic strategies were reported to reduce amyloid deposition, gliosis and tau hyperphosphorylation, rescuing cognitive and memory deficits in AD transgenic mice (Shi et al., 2011; Tweedie et al., 2012). In addition, intrinsic anti-inflammatory properties have also been attributed to TUDCA, which may explain the striking results obtained for astrocytosis and microgliosis in our model. TUDCA has been shown to decrease glial activation and microglial migration in acute neuroinflammation models both *in vivo* and *in vitro* (Yanguas-Casas et al., 2014). Finally, TUDCA has also been reported to inactivate GSK3β and reduce inflammation in liver tissue obtained after partial hepatectomy (Ben Mosbah et al., 2010).

Other studies also showed the anti-inflammatory properties of bile acids similar to TUDCA, such as ursodeoxycholic acid and GUDCA, in astrocytes and microglia exposed to pro-inflammatory stimuli including Aβ (Joo et al., 2003; Fernandes et al., 2007). Since Aβ accumulation is the main mechanism involved in glial activation during AD (Radde et al., 2006; Meraz-Rios et al., 2013), and GSK3β is also potentially involved, we suggest that TUDCA-mediated effects on Aβ load, GSK3β activity and TNF-α expression are responsible for the reduced neuroinflammatory conditions in TUDCA-treated APP/PS1 mice. In this regard, we are currently elucidating the molecular mechanisms by which TUDCA protects from glial activation.

Activation of PI3K/Akt signalling is also crucial for the expression of late-phase long-term potentiation (LTP), a form of synaptic plasticity that is closely related with

learning and memory, and specifically affected in AD (Selkoe, 2002; Horwood et al., 2006). This effect is particularly important in the dentate gyrus of the hippocampus (Karpova et al., 2006). Impairments in LTP may eventually culminate in synaptic depression that in turn is correlated with structural changes in synaptic morphology (Selkoe, 2002). Aβ oligomers also inhibit LTP and destabilize synaptic connection (Selkoe, 2002). Importantly, treatment with TUDCA resulted in a reduction of presynaptic terminals loss, as evidenced by an increase in the presynaptic marker synaptophysin in the dentate gyrus of APP/PS1 mice. This effect probably derives from the decrease in Aβ load, although activation of PI3K/Akt cannot be excluded. However, only a trend to improved spatial memory was detected in TUDCA-treated APP/PS1 mice compared to control APP/PS1 mice. Since this mouse model already presents extensive cognitive deficits and memory impairment at 7-8 months of age (Radde et al., 2006; Serneels et al., 2009), it is likely that TUDCA treatment started at 7 months is not sufficient to revert emerging or pre-established cognitive deficits. Still, mouse models do not develop the same type of cognitive decline usually observed in AD patients (Webster et al., 2014), which may also influence the behavioral data.

In conclusion, our results demonstrate the therapeutic efficacy of TUDCA in APP/PS1 mice with established amyloid pathology by attenuating Aβ production and deposition, tau pathology, glial activation and loss of synaptic function. Most of these effects are likely related to the activation of the Akt/GSK3β signalling pathway. However, modulation of Aβ deposition may influence several pathways that impact on tau hyperphosphorylation and neuroinflammation, which implicates that the reduction observed in Aβ load after TUDCA treatment may alter these AD-phenotypic traits by GSK3β-independent pathways. Finally, since chronic neuroinflammation is also strongly associated with accelerated AD progression, the anti-inflammatory properties of TUDCA further highlight its therapeutic potential. These evidences, allied to its safety and brain bioavailability, point TUDCA as a promising therapeutic strategy to attenuate AD progression.

V. CONCLUSION AND FUTURE PERSPECTIVES

The present work further expanded previous *in vitro* and *in vivo* studies regarding the therapeutic potential of TUDCA in AD. Here, we demonstrated that TUDCA treatment administrated to APP/PS1 mice after the onset of amyloid pathology decreases Aβ production and deposition, tau hyperphosphorylation and gliosis, while partially protecting against synaptic loss. Importantly, several of these effects might be connected to modulation of the Akt/GSK3β pathway. These results support the view that TUDCA can act as a multi-targeted strategy to treat several pathologies associated with AD progression.

Still, further work is needed in order to clarify the full range of TUDCA therapeutic targets.

One of the main questions concerns TUDCA-dependent modulation of the amyloidogenic processing of APP. Regarding this question, we are currently engaged in the optimization of a murine neuroblastoma cell model that overexpresses mutated APP to further evaluate the effect of TUDCA on the proteolysis of this protein. First, we intend to determine whether TUDCA modulates the intracellular trafficking of APP and its cleavage fragments by performing subcellular fractionation techniques, which could hint at the cellular compartment involved in altered APP processing (Lee et al., 2003). We hypothesize that TUDCA may modulate APP processing through the alteration of cellular cholesterol content or membrane fluidity, which would alter lipid raft content and thus APP trafficking and subcellular co-location with BACE1 (Marquer et al., 2011). Moreover, we intend to evaluate the effect of TUDCA on PI3K activation, a heterogeneous group of related kinases that regulate vesicular trafficking and alter APP metabolism. For this purpose, several approaches could be employed to identify the PI3K isoform possibly involved in APP processing, including different concentrations of the PI3K inhibitor wortmannin, which inhibits different PI3K isoforms depending on their subcellular location (Petanceska and Gandy, 1999), and small interference RNA approaches designed to downregulate the various isoforms of both the regulatory and catalytic subunits of PI3K. Other downstream targets of PI3K/Akt signalling implicated in AD could also be evaluated, especially mTOR.

Other possible way for TUDCA mode of action may rely on its anti-inflammatory properties. It would be interesting to further characterize the reactive phenotype of microglia beyond the pro-inflammatory cytokines tested in this study, focusing on M1

and M2 activation markers (Mandrekar-Colucci and Landreth, 2010; Meraz-Rios et al., 2013). Moreover, alterations in microglial phenotypic activation can lead to different Aβ clearance strategies, which could be determined by co-immunostaining for Aβ and a microglial marker. Also, the levels of Aβ-degrading enzymes insulysin, neprilysin and matrix metalloproteinase 9 should be assessed to study extracellular Aβ degradation (Hickman et al., 2008). Finally, GSK3β is a key mediator in glial activation, and further studies should be designed in order to dissect the role of TUDCA-dependent modulation of GSK3β and downstream inflammation-related pathways in both microglial and astroglial cultures exposed to several aggregated forms of Aβ. Co-cultures containing glial and neuronal cells could also be used to study possible cellular interactions and/or secretion of altered extracellular signalling molecules (Koistinaho et al., 2011; Ko et al., 2014).

The anti-apoptotic properties of TUDCA in AD remain to be studied *in vivo*, since the AD mouse model used in this study does not present neuronal loss at the time studied (10 months of age). In this regard, administration of TUDCA until later time points, during which neuron loss is detected in the dentate gyrus, would allow for the evaluation of the effect of TUDCA on Aβ-mediated apoptosis. Also, other AD mouse models could be engaged in this objective, such as the APP^{SL}/PS1KI double-transgenic mouse model, which is characterized by plaque deposition at 2 months of age, as well as high levels of intraneuronal Aβ, which correlates with elevated neuronal death in AD mouse models (Casas et al., 2004; Wirths and Bayer, 2010). Importantly, this transgenic model displays massive neuronal loss in the hippocampus at 6 months, along with memory deficits and synaptic dysfunction (Casas et al., 2004). Additionally, this AD mouse model could also be used to study the effect of TUDCA on mitochondrial dysfunction following Aβ accumulation inside this organelle, due to the presence of high intraneuronal Aβ levels of Aβ intraneuronally. Finally, double mutant APP/tau transgenic mice would also constitute an interesting approach, due to the significant neuronal loss observed at 9 months of age, the age of onset of amyloid deposition (Ribe et al., 2005). The added value of this model is the strong development of neurofibrillary degeneration features, which would allow for the study of TUDCA-dependent downregulation of GSK3β in the context of pathogenic tau hyperphosphorylation.

Finally, there are several pathological mechanisms in AD that span most neurodegenerative disorders, such as protein aggregation, neuronal death, mitochondrial dysfunction, oxidative stress and, specially, chronic neuroinflammation. Importantly, TUDCA stands as a promising agent capable of targeting several of these common pathophysiologic pathways shared among neurodegenerative diseases, which are desperately lacking appropriate therapies.

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